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***“Effect of Sugar vs Mixed Breakfast on Plasma Ghrelin
and P300 Potentials”***

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ABSTRACT

Background: Obesity is a health emergency growing worldwide. The hallmark of obesity is an augment in visceral fat. Alongside waist circumference gain, impaired blood lipid profile and hypertension it leads to Metabolic Syndrome (MetS). Amongst the several factors contributing to its spread, breakfast consumption plays a role promoting ghrelin regulation. In addition, breakfast consumption may ameliorate cognitive performance.

Aim: We focused on how three different commercial breakfasts, with different contents of macronutrients, affected: 1) metabolic response and sense of satiety via ghrelin regulation (protocol M); 2) cognitive processing of sensory information via P300 recording (protocol N). To our purposes, we administered three different commercial breakfasts and glucose, as control, to 12 healthy subjects (6M/6F; 22.2 ± 1.5 Kg/m²; 27 ± 5 yo) who underwent two different experimental protocols to assess aim 1 and 2. Aside control (**A** 50gr glucose) The commercial breakfasts were as follows: **B1**) milk (125mL) and cornflakes (30gr); **B2**) milk (220mL), apple (200gr) and chocolate cream filled sponge cake (30gr) and **B3**) milk (125mL), apple (150gr), bread (50gr) and hazelnut-chocolate cream (15gr).

Protocol M: each subject went through one Oral Glucose Tolerance Test (50gr-OGTT) and three-meal test loads (one for each tested breakfast). We collected blood samples at -15'; 0; 15'; 30'; 45'; 60'; 75'; 90' and 120'. Volunteers ate breakfast after the second blood sampling. We assayed glucose, insulin and ghrelin plasma levels (total).

Protocol N: in four different days, each subject underwent eight evoked related potentials measurement (ERPs), two for each breakfast (one after a night fast and one three hours after breakfast consumption). To our purposes, we recorded frontal zone (FZ) ERPs latencies.

Results: metabolic data showed that all three commercial breakfasts gave a significant lower glycemic response when compared to glucose (B1: $p < 0.05$; B2, B3: $p < 0.01$) alongside a lower insulin response (only breakfasts B1 and B2 in a significant fashion: $p < 0.01$). Ghrelinemia inhibition tended to be greater in B3 group (332.1 ± 30.2 pg/mL) with respect to A (420.6 ± 44.2 pg/mL), but did not achieve statistical significance. Electrophysiological data showed that, three hours after ingestion, B1, B2 and B3

displayed a decrease in FZ ERPs latency when respect to A, thus only B3 in a significant way ($p < 0.01$).

Conclusion: we demonstrated that different breakfasts were able to elicit lower glycemic and insulin responses even with a greater carbohydrate content. B1, B2 and B3 groups displayed a tendency to maintain satiety via ghrelin inhibition. Furthermore, hazelnut-chocolate cream breakfast (B3) consumption decreased P300 latency, suggesting ameliorated cognitive performance.

1. INTRODUCTION

A diet rich in high glycemic index foods is associated with a major risk of developing obesity and type 2 diabetes mellitus (T2DM). During the last 20 years, obesity and overweight incidence rose both in adult and in pediatric population. This tendency, spread from developed to developing countries leading to a world pandemic emergency so that, according to WHO data, obesity is responsible for at least 2.8 million deaths/year [1].

A Body Mass Index (BMI) greater or equal to 30 Kg/m^2 defines pathologic obesity in adults [2]. Increased BMI, alongside with augmented visceral fat, waist circumference gain, dyslipidemia and hypertension characterizes the Metabolic Syndrome (MetS) which is the major risk factor of one of the leading causes of death: cardiovascular diseases (CVDs). Obesity relates both to CVDs development and to overt T2DM. It is common to refer to diabetes and obesity as “diabesity”, to mark further the liaison between the two diseases [3].

Spread and incidence augment of obesity and T2DM are, in fact, parallel and both diseases show defects in insulin action (sensitivity and secretion). The molecular mechanisms that link obesity to diabetes are mainly due to the increased plasma Free Fatty Acids (FFA) levels typical of MetS. The higher amount of these molecules found in obese and overweight patients is due mainly to the particular food intake of this individuals and the augmented lipolysis activity [3].

It is known that adipose tissue is more active in overweight and obese subjects than healthy ones [3]. This augmented activity leads to increased FFAs levels. The subsequent augment in mitochondrial β -oxidation is able to interrupt glucose metabolism and to reduce its uptake and glycogen synthesis from the skeletal muscle. The constant state of hyperglycemia and the compensatory hyperinsulinemic effort by pancreas impair insulin sensitivity. This phenomenon, commonly referred as “glucotoxicity”, leads to glycation of proteins and the synthesis of advanced glycation end products, which may induce β -cell failure in secreting insulin and final stage of this exposure is β -cell apoptosis.

Furthermore, when fat intake from dietary habits increases, fat storage occurs in liver, skeletal muscle and β -cells. Since it is not common for these tissues to store high fat amounts, the excessive mitochondrial production of toxic reactive lipid species speeds up insulin resistance and impaired glucose metabolism. This leads, with time, to overt type

two diabetes mellitus. The liaison between obesity and diabetes is so strong that 90% of T2DM patients are also obese [4].

Overweight and obesity management costs, their comorbidities and social reflections led local authorities to develop new strategies to fight these diseases. However, despite efforts, knowledge on obesity pathophysiology is incomplete and a control on obesity incidence is still far. According to a survey from Eurostat, overweight and obesity occur in almost every Member State in European Union both in female and male population with rates that span between 37-56.7% and 51-69.3%, respectively. These obese subjects develop the above-mentioned typical features of MetS, which may lead to T2DM, CVDs, hypertension and some types of cancer [5].

Since MetS is associated with several and severe diseases, it is important to make efforts to prevent it, especially at young age.

MetS settles thanks to more than a few factors: social behavior, environment, genetic and metabolic features and physical inactivity. The range of cofactors responsible for overweight, obesity and MetS is so wide that even gut micro biome plays a role in these diseases onset [6]. This large pool of factors reflects the several therapeutic approaches to treat this condition: life-style modifications, diet, surgical interventions, counselling and so on.

As long as regarding alimentary behavior, excessive or imbalanced food intake leads to a disproportion in introduced/consumed energy equilibrium, causing visceral fat deposition. For these reasons, physicians intervened on diet and life-style in order to make energy balance healthy again for patients displaying MetS. However, an aspect concerning food, which received more and more attention during these years, exists: i.e. the effects of specific nutrients presents in food on pathophysiological and physiological events. Combined with meal caloric composition and nutritional facts, “eating patterns” affect gain weight. “Eating patterns” include food intake habits e.g.: frequency of meals and snack consumption [7].

Regular ingestion of meals helps preventing obesity predisposition and settling. Amongst the different meals of the day, breakfast plays a major role in energy homeostasis with its contribution both in caloric and nutrient intake [8]. A variety of studies conducted both in Western and Eastern countries report that breakfast skipping is associated with weight gain and obesity [8]. What is interesting is that these studies allow to state that the

association between breakfast skipping and weight gain is true regardless the existence of several factors that contribute to overweight and obesity, such as social, cultural and economic background.

This phenomenon may find explanation in the lack of satiety during the day and the following increased energy intake. Furthermore, a difference in post-prandial insulin levels subsists amongst the different eating patterns regarding breakfast consumption habits. People having breakfast regularly and people who skip it on a daily basis do not share the same post-prandial insulin levels. In fact, the latter category displays a higher insulin secretion after meal ingestion. In this condition, insulin may help fat storage [9].

Breakfast skipping is an insalubrious dietary habit in healthy individuals, obese patients and especially in children and adolescents. This use in the two latter classes associates to obesity predisposition and it is one of the causes of MetS incidence augment, a dramatic condition at young age that may affects development, social life and school achievements [8]. Indeed, the importance of breakfast relates not only to overweight and obese predisposition. Another aspect, which relates to all age classes, and better to children and adolescents, is that breakfast consumption helps cognitive functions [10-11].

Benton et al report that missing breakfast negatively influences memory [12-13]. This effect is reversed by glucose consumption. Even if the positive effect of glucose ingestion is powerful, it is too rapid to have a practical and prolonged influence on memory during morning. On the other hand, foods slowly releasing carbohydrates improve attention in late morning hours [14].

To stress out more the importance of breakfast at early age, evidences exist that first meal consumption improves attendance and level of achievements reached by students [12-13]. This is partly due to the known evidence that children need more glucose than adults do. In fact, starting from 4 years old glucose need in a child brain grows until it reaches a peak around 9 years old of age in which the need is four-fold higher. This condition decreases until 16-18 years old [15-16].

Neuronal effects of different breakfasts may vary because of the different macronutrients composition and those macronutrients relates to children behavior during school stay. Additionally, malnourished children seem to benefit from lower effects of breakfast consumption, as regarding attention issues [17].

Taken all together, these data show that breakfast consumption is important for at least two aspects: a better control on body weight and ameliorate attention, especially in children and adolescents. Furthermore, many children and adolescent present of skipping breakfast just because they do not want to do it. This population is more likely to be more physical inactive and to have a lower cardio respiratory fitness level. [18]

In this study, we focused on the above-mentioned two important aspects of breakfast consumption: satiety regulation and cognitive processing of sensory information. We investigated how breakfast consumption may affect satiety via ghrelin inhibition and its impact on cognitive processing on healthy, young individuals via P300 recording. Ghrelin, hormone mainly secreted by X/A cells in the stomach, shows several physiological activities. Amongst these, ghrelin has a role in sense of satiety and food carving behavior. P300 belongs to the Evoked related brain potentials (ERPs) waveforms. These scalp neural manifestations are widely used to assess cognitive performance. The reasons that led us to measure ghrelin inhibition as a biomarker of satiety and P300 recording as marker of cognitive performance are explained further in this thesis.

With this in mind, we administered three commercially available breakfasts to 12 healthy, young volunteers, evaluated their metabolic and neural responses and compared them with our control, identified as 50gr of glucose.

1.1 Ghrelin

Ghrelin is a peptide hormone first described in 1999 by Kolima et al [19]. Its principal functions regard food intake and meal initiation. The X/A like cells in stomach mainly secrete ghrelin. These cells are copious in the oxyntic mucosa, particularly frequent in the gastric fundus and their number decreases ascending to the pylorus. Pancreatic islet cells display ghrelin and its receptors too, as part of evidence of its function in regulating glucose homeostasis but literature shows discrepancies about this feature [20].

Instead, a strong evidence in published studies suggest a role of ghrelin as energy storing promoter. In fact, ghrelin plasma levels augment before a meal intake and after a long period of negative energy balance (i.e.: a state in which the energy consumed is higher than the energy introduced via alimentation) [21-22]. As an example of this evidence: subjects suffering from anorexia nervosa display higher levels of plasma ghrelin when compared to subjects with a normal Body Mass Index (BMI) [23]. Vice versa, in obese patients ghrelinemia is lower but does not show down regulation after food intake [24], which should be ghrelin usual pattern after food consumption [25].

Ghrelin physiological actions stress out the “energy storing promoter” hypothesis since, in addition to meal initiation properties, ghrelin is also responsible for igniting research of food behavior. The hormone stimulates craving for a particular quality of food eaten: studies have shown that high ghrelin plasma levels augment the intake of food rich in fats and energy density [26]. In addition to high fat and energy content food craving, ghrelin promotes adipogenesis, lipogenesis and the usage of other nutrients as energy substrates rather than lipids [27]. Furthermore, ghrelin is able to induce gastric acid and digestive enzymes secretion and to promote bowel movements thus easing nutrients absorption and strengthening its function as energy storage promoter [28].

After secretion, ghrelin is rapidly acylated in its active form (acylated-ghrelin) and systemically reaches the brain and its target cells: the neurons in arcuate nucleus expressing ghrelin receptor, GHSR1a [29]. After GHSR1a binding, intracellular calcium rapidly increases, thus activating calmodulin kinase-kinase 2 (CaMKK2), which in turn starts a phosphorylation cascade leading to neuropeptide Y and agouti-related protein (NPY and AgRP) transcription [30]. Further details of ghrelin actions after binding to GHSR1a receptor are described in *figure 1.1a*.

Due to the functions ghrelin exerts on energy homeostasis (triggering food craving, high-energy content foods ingestion and fat storage), research focused more and more on ghrelin and its possible implications in obesity treatment. For these reasons, we introduced ghrelin measurement in study design, in order to clarify better the role of different tested breakfasts' consumption in satiety regulation.

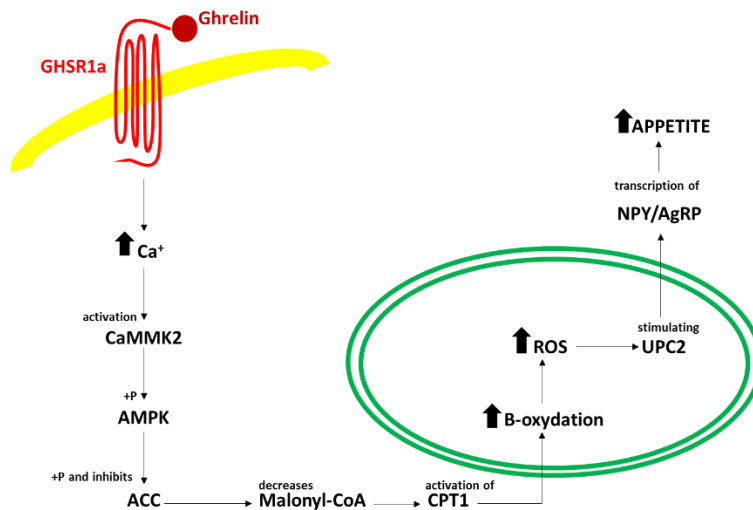


Figure 1.1a: schematic representation of ghrelin molecular mechanisms in cells expressing GHSR1a. CaMKK2: calmodulin kinase-kinase 2; +P: phosphorylation; AMPK: adenosine monophosphate-activated protein kinase; ACC: acetyl coenzyme A carboxylase; CPT 1: carnitine-palmitoyltransferase-1; ROS: reactive oxygen species; UCP2: uncoupling protein-2; NPY: neuropeptide Y; AgRP: Agouti-related protein.

1.2 P300 Event Related Brain Potentials

Mechanisms of evolution developed the ability to drive proper reactions to external stimuli by telling apart amongst what is novel or unexpected and what is not. In this way, we are able to generate a natural and quick response to what could be a possible danger. Event-related brain potentials (ERP) are scalp manifestations of neural activity and reflect this shift of attention focus caused by the influence of certain events on consciousness [31]. Sutton et al. first described these modifications in electroencephalogram voltage in 1965 [32]. This particular waveform latency peak is between 250ms and 350ms, so the event-related potential generated is usually referred as P300 or P3. This text refers to it as P300.

Since its finding, P300 was used to monitor and assess cognitive performance. P300 recording became a widely used test to evaluate this feature and several recording techniques relate to new stimulus response: e.g.: skin conductance, pupillary dilatation, scalp or intracranial recorded and magnetic resonance imaging (MRI). After discovery, this safe protocol has been tested on children, elderly and brain-damaged patients [33-34].

The detachment between “danger” and “no danger” after an exposure to an external stimulus relates to context. David Friedman et al [35] wrote a reference that gives a simple and clearly understandable example involving an everyday life situation: a car driver and a pedestrian perceive car honks in a different way, which elicit different reactions. In fact, while driving it is usually common to listen to external noises (e.g.: music or engine sound) in background but, if a new or unexpected sound is heard (a car horn) the attention will shift to the source of the new sound, thus generating an *orienting response*. The latter, defined as a rapid, involuntary shift of attention, appears to be necessary for survival [36-37]. Orienting is rapid and allows the subject to become aware of a possibly dangerous situation and to activate particular behaviors. As an evidence of the biological significance of this response, three months old babies orient their attention when exposed to the onset of a female voice [38].

ERPs are electrical activity manifestations generated by neuronal activation localized in different scalp regions and their name, event-related, clearly identifies their nature since these potentials are elicited by specific events. Their biological function is to orient the subject’s awareness to something requiring an evaluation, in order to avoid or affront a

potentially dangerous situation. The above-mentioned example could be useful again: if a driver hears a car horn, he orients its attention towards it in order to evaluate its source and everything else related (a dangerous, approaching car or a car in another route, honking to a distracted pedestrian).

We recorded ERPs in our study since their use as a tool of cognitive performance evaluation. This recording technique is widely used in assessing cognitive performance in subjects suffering from Parkinson and Alzheimer diseases [39]. Moreover, metabolic state and appetite may interfere with P300 evoked related brain potentials thus affecting cognitive performance [40].

Three dimensions define ERPs waveforms: scalp region, amplitude and latency. The scalp regions relevant for our study and depicted in figure are Frontal (FZ); Central (CZ) and Parietal (PZ) (*figure 1.2a*). According to several studies, which managed to separate P300 waveform components, to evaluate cognitive performance recording should occur in FZ scalp region [41]. Amplitude is the extent of neural activity and latency, measured in milliseconds (ms) is the point at which the amplitude of a particular waveform is maximum. As previously stated in this text, P300 waveform amplitude reaches its maximum between 250 and 350ms, thus the origin of the name.

Relating to the previously described driver-car horn example it is easy to notice that not every external or “deviant” stimulus redirects the focus of attention: not every sound heard while driving is able to orient the attention from one focus to another. In fact, P300 is not evoked by every external stimulus [42]. Throughout the first 100ms after stimulus exposure, an early activity exists. This activity, the mismatch negativity or MMN [42], reflects the fact that the brain has found something different, new or unexpected in between the normal stream of “standard” stimuli. MMN echoes the setting of a comparator mechanism.

This machinery is evoked by every event capable of subtracting the focus of attention by the main task (e.g.: watching a movie, driving a car or reading a book) to the deviant stimulus. However, even if it is true that every deviant event may elicit a MMN comparator answer, it is not true that following every MMN the P300 occurs i.e.: not every deviant stimulus generates an orienting response.

Gaeta et al. and Schröger [43-44] have firstly described this. Their evidences support that, in order to generate the following P300, MMN has to be sufficiently large. In other words,

the event responsible of MMN settling has to be sufficiently deviant from the environment. Furthermore, their studies put a light on another aspect of ERPs: MMN and subsequent P300 generation occurs only if the deviant stimulus is presented in-between the recurrence of repeating standard stimuli. In fact, MMN does not occur if the deviant provocation lays at the start of the experiment [45]. This underlines the fact that P300 is a response to something new experienced by the brain during a situation classified as “normal” or “standard”. These evidences reinforce the hypothesis that P300 is a reaction to new stimuli.

In addition, and hence strengthening the former evidence, Sokolov et al [46] have demonstrated that the answer to deviant stimuli was no longer elicited after repeated presentation. This finding suggests a memory for the repeated events. The biological meaning of this adaptation finds partly explanation by the need to orient the attention only towards what is really new and deviant.

One can use several experimental methods to evoke and then measure P300 waveforms. In a typical experimental set up, two classes of stimuli exists: the *standard* one and the *deviant* one. While the former is a repetitive stimulus to which the subject undergoing the measurement is exposed, the latter is the culprit of potential evocation and usually referred as “the oddball” event. The stimuli used can have different sources: visual, somesthetic or auditory [47-48].

During these experiments, subjects follow a specific task while being exposed to the target stimulus. The task may be *active*, *passive* or *novelty oddball*. In *active* tasks, subjects receive instructions to count mentally the events or proposed stimuli.

While performing *passive* tasks, volunteers’ attention is driven away from focus (e.g.: reading a book or watching a silent movie) by a deviant stimulus. In *novelty oddball* tasks, participants experience three stimuli with different frequencies: one high probability standard stimulus and two low frequency deviant ones.

As tasks, stimuli belong to different classes as well: these stimuli can be somesthetic, visual or auditory. In our study, as in the majority of reported experiments [32-48-49], we used auditory stimuli.

Studies prove that P300 waveform is impaired in patients showing lesions on the frontal or prefrontal region [50]. Other studies have subsequently proven via functional magnetic

resonance imaging (fMRI) that frontal scalp regions express P300. Due to the selective expression in frontal region of the scalp, we used for analysis FZ recorder potentials.

Merging the evidence until now found in literature, the essential features that a particular stimulus has to have, in order to be able to generate an orientating response, are the following:

- Unexpected;
- New;
- Unpredictable;
- In-between something new or experienced.

We used these characteristics to design the experimental protocol used in this study, which is an active task. Both the standard and the deviant stimuli were auditory. Material and methods section discuss standard/deviant ratio and the detailed protocol.

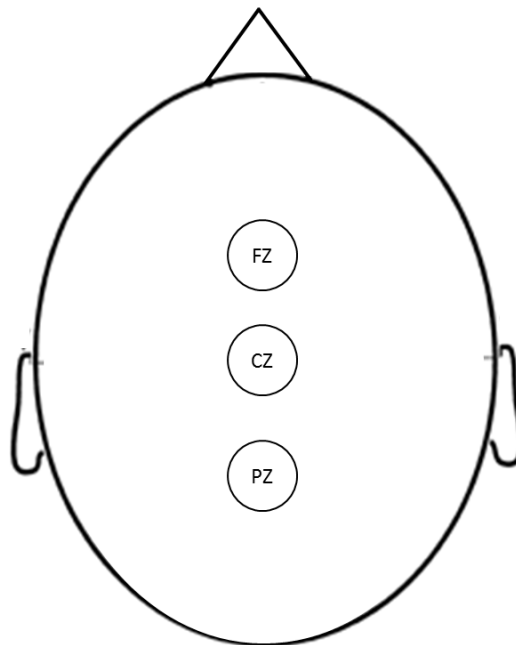


Figure 1.2a: schematic representation of the scalp region of interest during our P300 recording. FZ: Frontal Zone; CZ: Central Zone; PZ: Posterior Zone. For our purposes, FZ potentials were analyzed.

2. MATERIAL AND METHODS

2.1 Subjects

We enrolled twelve volunteer subjects from “Università degli Studi di Milano” students or IRCCS Policlinico San Donato employees. The male to female ratio was 6/6; BMI and age were as follows: $22.2 \pm 1.5 \text{ Kg/m}^2$ and 27 ± 5 years old (mean \pm standard error of the mean).

All subjects were healthy and were eligible to access the study according to the inclusion/exclusion criteria presented in **table 2.1a**. To maintain the same metabolic profile during the duration of the study, each volunteer received instructions not to change his or her dietary lifestyle. Experimental protocol received positive consent by “Università degli Studi di Milano” Ethical Committee and informed written consent was obtained from each volunteer in accordance with Declaration of Helsinki guidelines. All procedures used complied with the Good Clinical Practice (GCP) principles.

<i>Inclusion criteria</i>	
Age	18-40 years old
BMI	18.5-24.9 Kg/m ² according to NHLBI
	Stable diet
<i>Exclusion criteria</i>	
	Type 1 or type 2 Diabetes (ADA criteria)
	Glucose intolerance (ADA criteria)
	Dyslipidemia (ATPIII according to NCEP)
	Metabolic Syndrome

Table 2.1a: table reporting inclusion and exclusion criteria evaluated for this study. BMI: Body Mass Index; NHLBI: National Heart, Lung and Blood Institute; ADA: American Diabetes Association; ATP III: Adult Treatment Panel; NCEP: National Cholesterol Education Program.

To establish the basal metabolic state of the subjects, they underwent a basal blood withdrawal to determine lipid basal profile (total cholesterol; LDL; HDL; triglycerides and free fatty acids), glucose and insulin plasma levels. We analyzed this profile during

the first visit to assess study inclusion/exclusion. All subjects tested for inclusion were eligible for the study, **table 2.1b** represents basal data.

	Mean	SEM \pm
<i>Glucose (mg/dL)</i>	84,6	1,9
<i>Cholesterol (total) (mg/dL)</i>	163,8	5,4
<i>HDL-C (mg/dL)</i>	63,3	2,3
<i>LDL-C (mg/dL)</i>	95,8	5,5
<i>Triglycerides (mg/dL)</i>	79,3	12,7
<i>Insulin (uU/mL)</i>	7,6	0,8
<i>NEFA (mmol/L)</i>	0,7	0,1

Table 2.1b: table representing basal data of the twelve subjects assessed for eligibility. HDL: High Density Lipoproteins; LDL: Low Density Lipoproteins; NEFA: Non-esterified fatty acids.

After formal admission into the study, the subjects underwent two protocols: one, referred as **protocol M** to evaluate metabolic effects and ghrelin inhibition and one, referred as **protocol N** to measure evoked related brain potentials. **Figure 2.1a** provides a schematic representation of study enrollment and design. Subjects participated to protocol M and protocol N in different days. The eight experimental days were separated at least by two weeks one from the other.

2.2 Breakfasts' composition

Breakfasts' composition and nutritional facts are reported in **tables 2.1c and 2.1d**, respectively.

A	B1	B2	B3
<i>Glucose 50gr</i>	<i>Milk 125mL</i> <i>Corn Flakes 30gr</i>	<i>Milk 220mL</i> <i>Apple 200gr</i> <i>Chocolate cream sponge cake 30gr</i>	<i>Milk 125mL</i> <i>Apple 150gr</i> <i>Hazelnut-chocolate cream 15gr</i> <i>Bread 50gr</i>

Table 2.1c: table reporting the composition of the three commercial breakfasts tested.

	A	B1	B2	B3
<i>Kcal</i>	190	170.5	329.2	350.5
<i>KJ</i>	795.5	724.8	1389.2	1481.2
<i>Proteins (gr)</i>	0	6.2	11	9
<i>Carbohydrates (gr)</i>	50	31.5	51.8	61.4
<i>-Sugar (gr)</i>	50	8.5	44.9	35.1
<i>Lipid (gr)</i>	0	2.2	9.5	8.3
<i>-Lipid (gr)</i>	0	1.4	4.7	3

Table 2.1d: table reporting the nutritional facts of glucose (A) and the tested breakfasts. B1) milk and corn flakes; B2) milk, apple and chocolate cream filled sponge cake; B3) milk, apple, bread, hazelnut-chocolate cream.

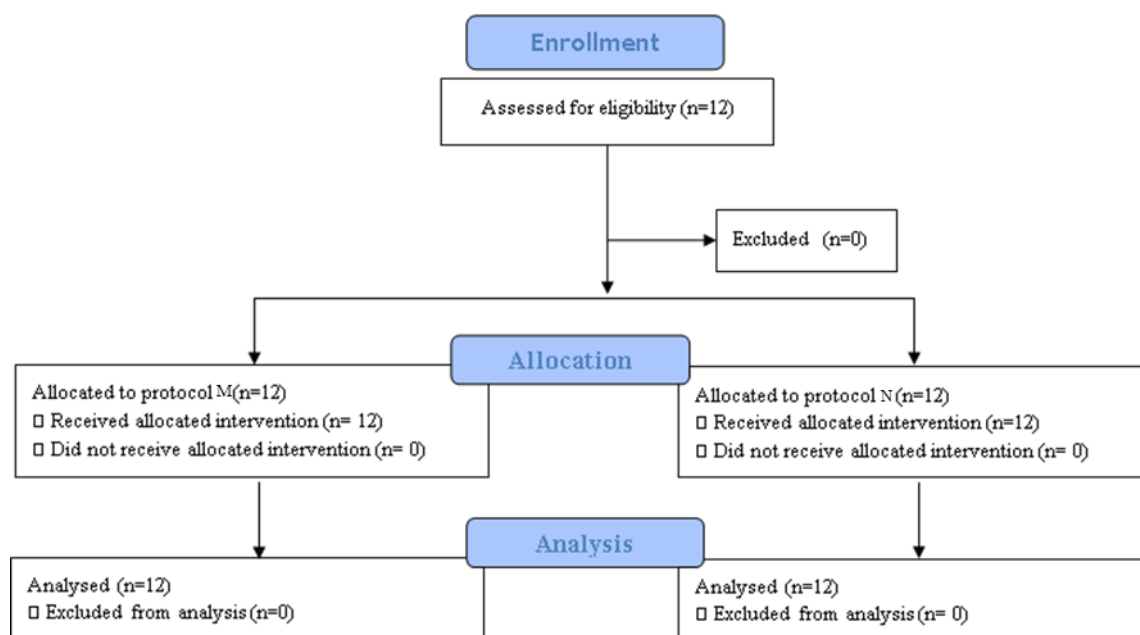


Figure 2.1a: Volunteers' enrollment and protocol allocation chart. No volunteer tested for eligibility was excluded. Every volunteer was allocated to protocol 1 and 2 and every data has been used for analysis.

2.3 PROTOCOL M-Oral Glucose and Breakfast Test Load

To assess metabolic and hormonal profile each subject underwent a 50gr-OGTT and three distinct Breakfast Test Loads (BTLs), one for each meal tested.

Subjects received instructions to fast overnight (12 hours) and to show up the day after to start the test, between 8:30 and 9:00 in the morning. After an acclimation time, adequate personnel inserted a cannula needle in the volunteer's forearm and took a first blood sample (time -15') consisting of two tubes: plasma and serum to test glucose, insulin and ghrelin hematic levels. 15 minutes after the first blood sampling, we took a second one (time 0) to have two basal data. Immediately after, volunteers drank the glucose diluted in aqueous solution or ate assigned breakfast within a given ten min time.

Then they underwent serial blood samplings until the end of the two hours of OGTT or BTLs (15'; 30'; 45'; 60'; 75'; 90'; 120'). During each experimental day, the total amount of blood withdrawn from volunteers was about 58.5mL. After an acclimation time, subjects were able to leave. This routine, was repeated for glucose (A) and test meals B1; B2 and B3 in experimental days separated by 2 weeks.

Blood samples were kept on ice until centrifugation, which occurred at 4° within one hour and a half after sampling. After centrifugation, samples were split in several aliquots and frozen at -80°C for ghrelin analysis and storage. Free-insulin was dosed by a highly specific 2-site monoclonal antibody-based immunosorbent assay (ELISA; Dako Diagnostics, Cambridgeshire, UK). Plasma ghrelin level (total) was measured through enzyme-linked immunosorbent assay (ELISA, Merck Millipore, Darmstadt, Germany).

As regarding immunosorbent technique, all samples were analyzed in duplicate, intra and inter assay variability were below 10%. Duplicate samples exceeding 10% CV, or samples which optical density (OD) did not fall between the linear range of the standard curve, were repeated.

2.4 PROTOCOL N-Cognitive processing of sensory information

Cognitive processing was assessed via the previously described P300 ERPs evaluation. As beforehand stated, the experimental set up used for our study was an active oddball task with auditory stimuli.

The standard stimulus was a “beep” sound while the deviant or oddball one consisted of a pitched “beep” sound. The subjects had to follow a simple, active task i.e.: mentally counting the number of pitched “beep” sounds heard through an earphone set and report it to the researcher.

Five electrodes were placed on the following scalp regions: the above-mentioned frontal zone (FZ); central zone (CZ); posterior zone (PZ) and A1-A2 regions. The latter, refers to Auricula zone 1 and 2, the left and right ear, respectively and served as signal stabilizer. FZ, CZ and PZ was instead used for signal recording. For further analysis, we used only FZ P300 latency. The electrodes were connected to neural machine Synergy (Lubiana, Slovenia).

Subjects received instruction to fast overnight (12 hours) and to show up the day after to start the test, between 8:30 and 9:00 in the morning. After electrode placement, measurements took place with subjects sitting and keeping their eyes closed, in order to avoid visual stimuli.

Each subject followed this routine on the same day:

- Admission at the hospital
- First ERPs recording
- Assigned breakfast consumption within 10 minutes
- Three hours waiting period
- Second ERPs recording

During both first and second Evoked Related Potentials recording, volunteers had to listen to repeated “beep” sounds (the standard event) and to mentally count different ones (the oddball event). In this occasion, oddball events were pitched “beep” sounds. The standard/oddball events ratio was 80/20%. We presented the oddball events in a randomized fashion, to keep the stimulus “new” and “unexpected”.

To obtain a valid ERPs measurement and to avoid the influence of other external stimuli, subjects kept their eyes closed during P300 recording.

By excluding visual stimuli, we were certain that recorded P300 arose after experimental provocation only.

This routine, was repeated for glucose (A) and test meals B1; B2 and B3 in experimental days separated by 2 weeks.

2.5 ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) is a laboratory technique that exploits protein property to adsorb to plastic surfaces and antibody-antigen reaction. Thanks to this technique, it is possible to detect a specific molecule recognized by a specific antibody and to quantify it by utilizing a standard curve.

ELISA can have different approaches in detecting the target molecule; the one used in this protocol is a sandwich-ELISA. This protocol aims to form an antibody-antigen-antibody complex, the “sandwich”, which can be detected via a streptavidin-Horseradish Peroxidase (HRP) complex. All reactions take place in a 96-flat bottom-wells microtiter plate, which is pre-incubated with an amount of specific antibody.

In every plate, unknown samples, controls and standard are added. Controls are samples in which the target molecule is known and are used to verify the good quality of the assay. While the purpose of controls is to determine the quality of the experimental run, standards function is to generate the curve that will be used to obtain concentration values. Standards are, in fact, samples containing a specific amount of target molecule. These samples' concentration varies from high to low following a specific dilution (e.g.: 1:10 or 1:2). The OD read from these samples will be the reference to create a standard curve. The OD read from the unknown samples is then interpolated to standard curve to calculate the amount of target molecule in samples. **Figure 2.5a** summarizes the protocol used.

Each sample is co-incubated in duplicate with a biotinylated antibody to form the sandwich complex as seen in figure 1. Subsequently, the most important step of ELISA procedure occurs i.e.: wash. This step is particularly critical since it allows the removal of unwanted molecules and the excess of antibody or reagents used during the protocol. Improper wash ends with inaccurate results, high CV% values or failure in color development. After a series of three washes with the wash buffer, HRP is added into the plate to bind to the biotinylated antibody. The excess of HRP is washed away with three washes. Again, this step is crucial to avoid improper results. The antibody-sample-biotinylated antibody-HRP complex adsorbed to the microtiter plate is incubated with TMB. This molecule is the substrate of HRP, which starts catalyzing the colorimetric blue reaction. After an incubation time, the reaction is blocked by adding 2N HCl.

The result is that blue color turns from blue to yellow and its intensity is proportional to the analyte present in the unknown sample. Via a reader, the microtiter plate OD is read.

A software builds the standard curve and, after samples' OD interpolation, gives the unknown samples concentrations.

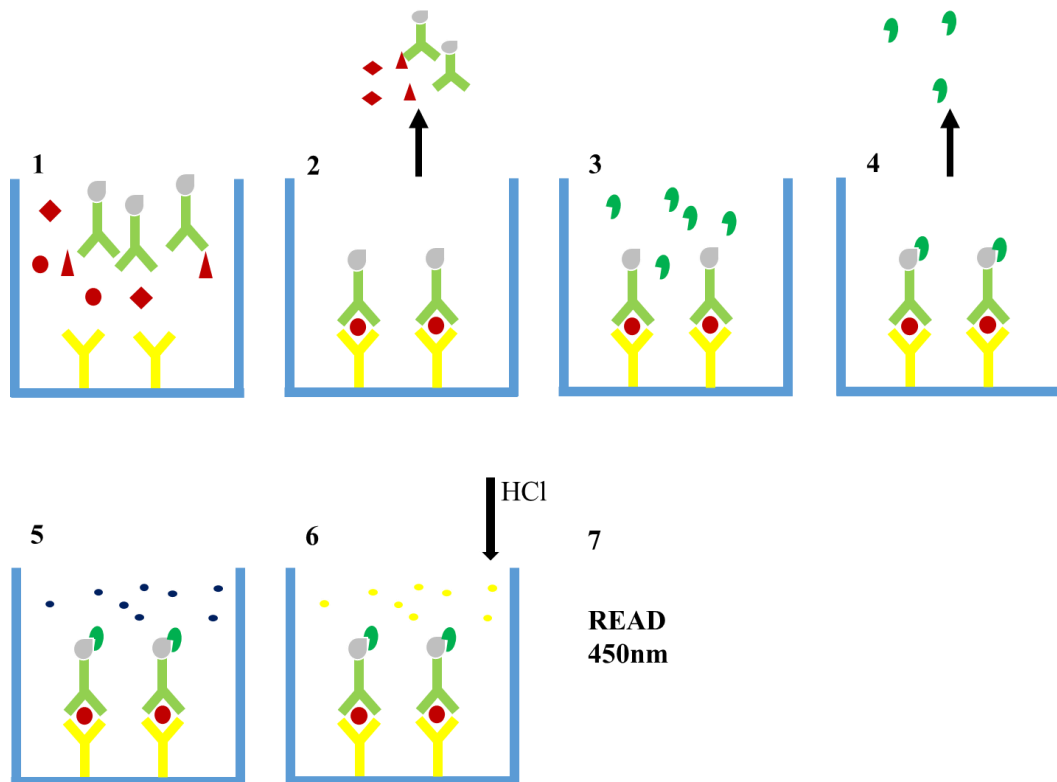


Figure 2.5a: Schematic representation of sandwich ELISA protocol: 1) an amount of the unknown sample (red dots, triangles and diamonds) and of the specific biotinylated antibody are added into the well to incubate; 2) sample and antibody are then removed via several washes; 3) HRP (green) is then added into the wells to bond with biotinylated antibody (grey); 4) HRP excess is washed away; 5) TMB substrate (blue dots) is added in order to start colorimetric reaction; 6) the reaction is then stopped with 2N HCl and 7) the plate is read at 450nm.

2.6 Area Under the Curve (AUC)

We used Area Under the Curve (AUC) to evaluate glucose, insulin and ghrelin excursions during OGTTs and BTLs. To calculate AUC we used the trapezoidal rule and the following method, described for OGTT, was used for every OGTT and BTLs.

For each experimental day, time course data of glucose were collected and used to build a graph as shown in figure. The purpose of AUC method is to separate the whole area underneath the graph curve in trapezoids, calculate their area and sum it. To obtain the areas, the height of each trapezoid has to be multiplied by the sum of the basis of it and the result divided by 2.

These dimensions are identified on the graph on y and x-axis: the height lays on x and it is the result of the subtraction ($x_1 - x_0$) while the basis lay on y and are identified as y_0 and y_1 . Dimensions are depicted in *figure 2.6a*.

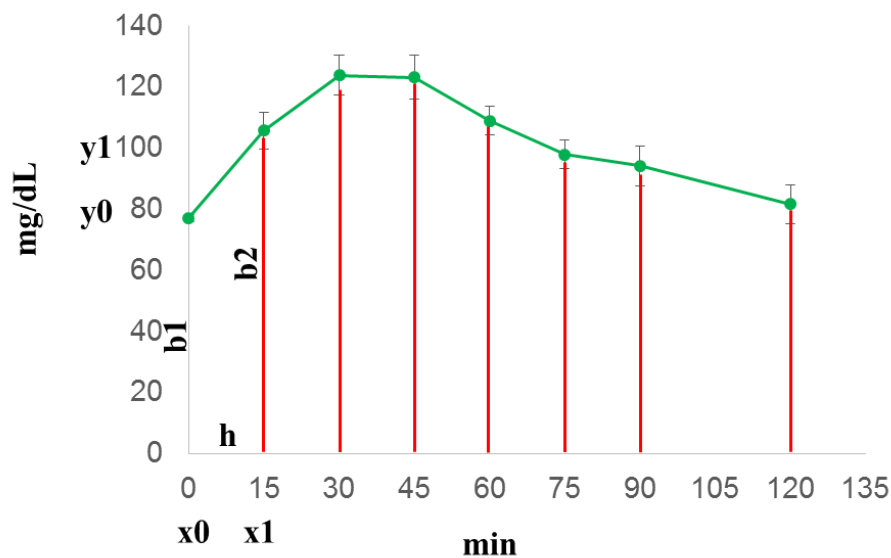


Figure 2.6a: Area Under the Curve AUC formula dimensions representation: the data needed to calculate the area of the first trapezoid to the left are: the time interval between the two measurements given by the subtraction ($x_1 - x_0$; 15-0); glucose concentration at time 0 (which is labelled as y_0); glucose concentration at time 15 (which is labelled as y_1). Height is labelled as “h”; b_1 and b_2 stand for base 1 and base 2, respectively.

The sum of each trapezoid AUC gives total AUC. Incremental AUC was obtained by subtracting the AUC of the basal concentration

(calculated from minute zero to minute 120) from the total AUC (*figure 2.6b*).

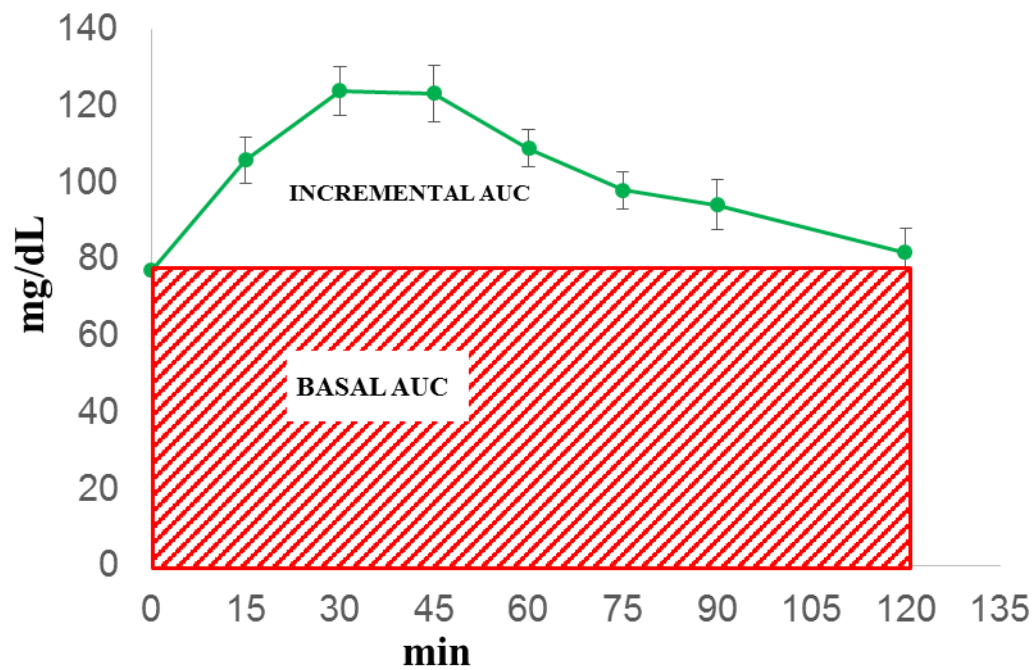


Figure 2.6b: Area Under the Curve AUC formula dimensions representation: the data needed to calculate incremental AUC. Basal AUC is obtained by using the depicted dimensions and it is represented by the red triangle. The result of subtracting basal AUC from total AUC is incremental AUC, which is the area in between basal AUC and the curve (green line).

2.7 Statistical Analysis

The comparison among studies was performed using within-subjects ANOVA with Greenhouse-Geisser correction followed by Dunnet's post-hoc tests (A, glucose as control). Pearson's correlation test and linear regression analysis were conducted between plasma ghrelin and FZ decrement.

All data were represented as mean \pm SEM. An α level of 0.05 was chosen as statistically significant. Analyses were carried out with the Statistical Package SPSS version 22 for Mac (Armonk, NY, USA; IBM Corp.), Excel 2011 (Redmond, WA, USA; Microsoft), GraphPad Prism 7 (San Diego, CA, USA).

3. RESULTS

3.1 Metabolic Data

To evaluate metabolic profiles in the different breakfasts and in glucose, we assayed total ghrelin, insulin and glycaemic serum levels during OGTT and BTLs. All data are represented as mean \pm standard error of the mean. **Table 3.1a** report all data.

3.1.1 Glycemia

All subjects exhibited a similar and within normal range fasting plasma glucose (77 ± 1.3 mg/dL). Glycemic curves for glucose and each breakfasts are depicted in. Glucose consumption alone displayed a greater augment in glucose plasma levels when compared to all three commercial breakfasts. This data is visually represented comparing the OGTT and the BTLs one with another (**figure 3.1.1a**); furthermore, the Area Under the Curve (AUC) for each experimental condition is significantly different from the control one (Repeated Measures ANOVA with Greenhouse-Geisser correction: $p < 0.0001$).

Dunnet's post-hoc test revealed that: A and B1 AUCs were higher than B2 and B3 and all three commercial breakfasts differed in a significative fashion from glucose AUC, thus in a different way (A vs B1: $p < 0.05$; A vs B2-3: $p < 0.01$).

Control glucose plasma level, reached a peak 30 minutes from ingestion had occurred (123.8 ± 6.5 mg/dL). When breakfasts were compared to control, B2 and B3 showed a significantly lower augment in glucose plasma level 30 minutes after ingestion (86.5 ± 4.6 mg/dL; 92.2 ± 5.1 mg/dL, respectively. $P < 0.05$). B1 plasma glucose concentration 30 minutes after ingestion was still lower than control (109.8 ± 4.2 mg/dL) but not in a significant fashion.

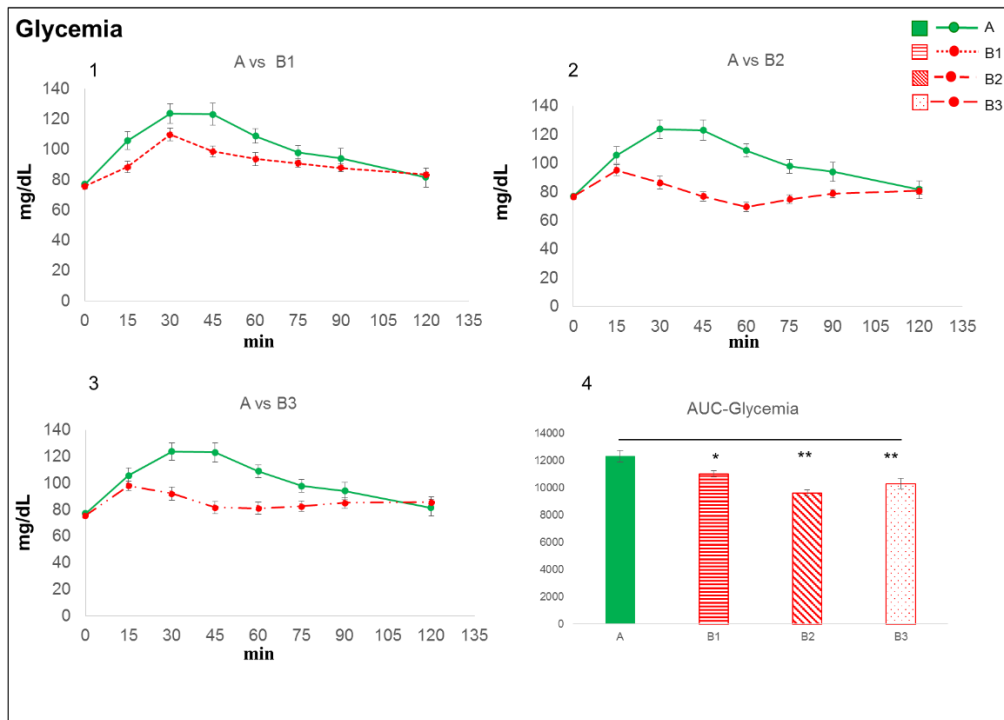


Figure 3.1.1a: glycemia excursions graphs. Graphs 1-2-3-4 show comparisons between glucose OGTT (green line) and every breakfast test load. Graph 1: glucose vs B1 (cereal and milk); graph 2: glucose vs B2 (apple, milk, chocolate cream sponge cake); graph 3: glucose vs B3 (milk, apple, bread, hazelnut chocolate cream); graph 4: area under the curve (AUC) comparison. Repetead Measures ANOVA with Greenhouse-Geisser correction was significant ($p < 0.0001$). Dunnet's post-hoc test revealed that B1 ($p < 0.05$) as well as B2 and B3 ($p < 0.01$) had lower glucose AUCs than Control. (All data are represented as mean \pm sem. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$)

3.1.2 Insulinemia

All subjects exhibited a similar and within normal range plasma insulin level ($5.25 \pm 1.49 \mu\text{U/mL}$) Plasma insulin levels obtained during experimental OGTTs and BTLs are represented in *figure 3.1.2a*. When compared with glucose, all tested breakfasts showed smaller insulin excursions.

Repeated Measures ANOVA analysis between the four groups resulted to be significant ($p > 0.001$). Dunnet's post-hoc revealed that B1 and B2 ($p < 0.01$), but not B3, had smaller insulin AUCs than control.

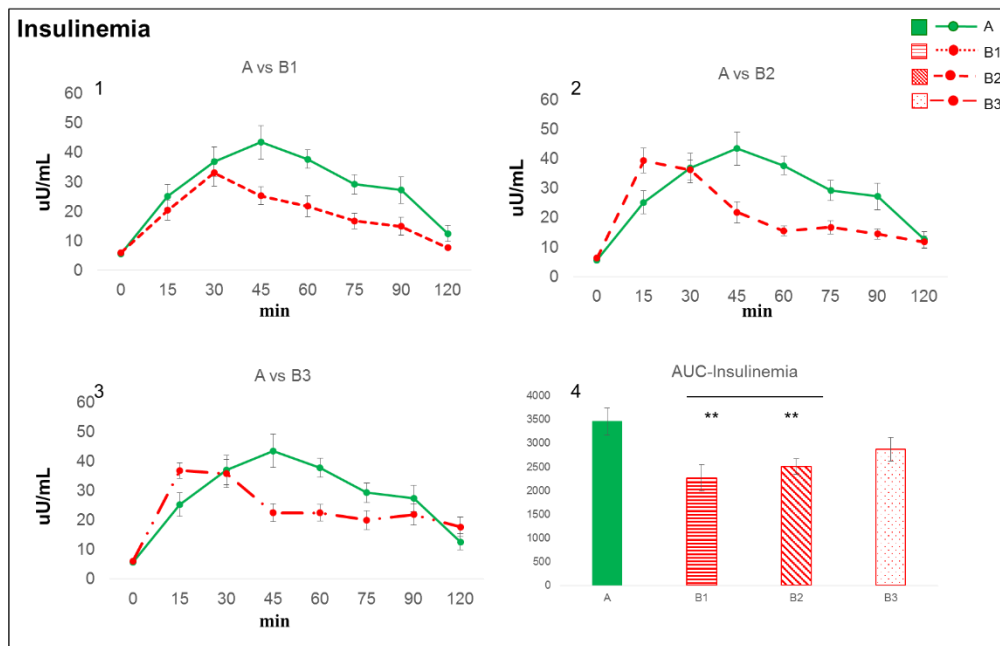


Figure 3.1.2a: insulinemia excursions graphs. Graphs 1-2-3-4 show comparisons between glucose OGTT (green line) and every breakfast test load. Graph 1: glucose vs B1 (cereal and milk); graph 2: glucose vs B2 (apple, milk, chocolate cream sponge cake); graph 4: glucose vs B3 (milk, apple, bread, hazelnut chocolate cream); graph 4: area under the curve (AUC) comparison. Repeated Measures ANOVA was significant ($p < 0.001$). Dunnett's post-hoc test revealed that B1 and B2 ($p < 0.01$), but not B3 had lower insulin AUCs than Control. All data are represented as mean \pm sem. *: $p < 0.05$; **: $p < 0.01$.

3.1.3 Ghrelinemia

Plasma ghrelin was similar at baseline (415.5 ± 5.2 pg/mL) in all subjects. After meal or glucose ingestion, 2-hour plasma ghrelin attained the lowest level in B3 (332 ± 30.2 pg/mL), but the decrement with respect to A (420.6 ± 44.2 pg/mL) did not achieve statistical significance. Final plasma ghrelin inhibition (data obtained by simply subtracting the ghrelin value at 120 minutes to the one at 90 minutes) tended to be higher in B1; B2 and B3 groups with respect to A, without achieving significant differences among the four tests (Repeated Measures ANOVA). **Figure 3.1.3a** depicts ghrelin plasma levels during OGTT and BTLs.

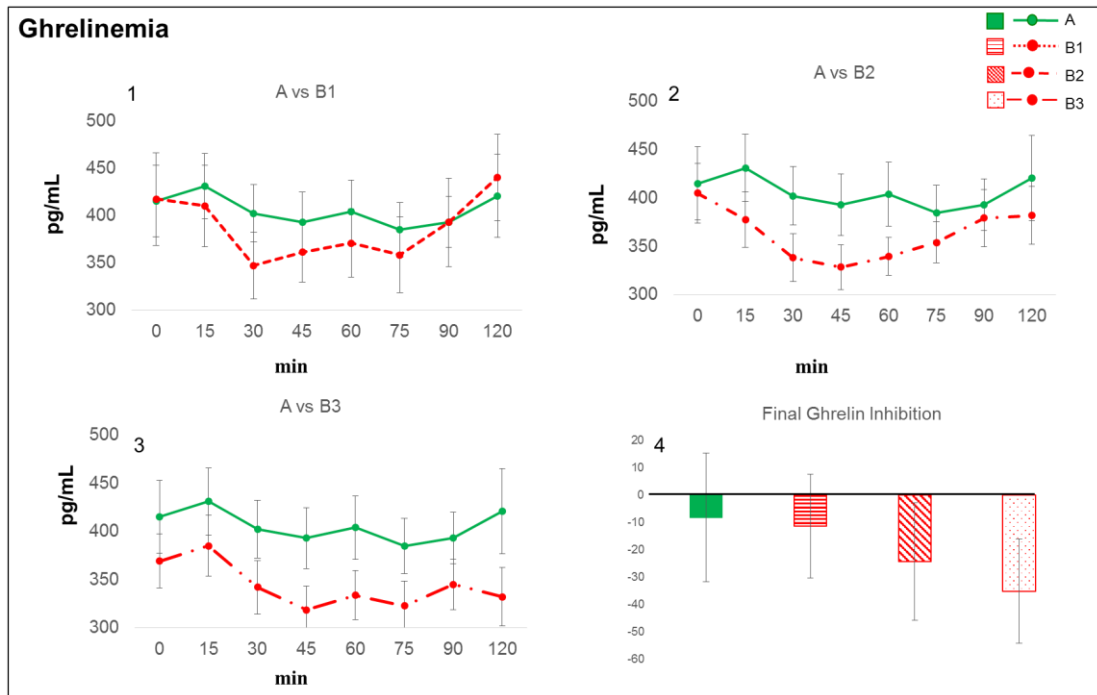


Figure3.1.3a: ghrelinemia excursions graphs. Graphs 1-2-3-4 show comparisons between glucose OGTT (green line) and every breakfast test load. Graph 1: glucose vs B1 (cereal and milk); graph 2: glucose vs B2 (apple, milk, chocolate cream sponge cake); graph 4: glucose vs B3 (milk, apple, bread, hazelnut chocolate cream); graph 4: area under the curve (AUC) comparison. Repetead Measures ANOVA applied to the final (90-120 min) level of ghrelin (expressed as change with respect to the baseline) found no significant difference among the four groups.. All data are represented as mean \pm sem.

3.2 Electrophysiological data

Three hours after ingestion, all of the tested breakfasts displayed a FZ decrement with respect to control A (*figure 3.2a*). The incremental frontal P300 potential (obtained by subtracting the latency value pre-ingestion from the latency post-ingestion) showed a significant anticipation only in B3 (-33.6 ± 5.9 ms) with respect to A (9.7 ± 6.6 ms). Repeated Measures ANOVA with Greenhouse-Geisser correction was significant ($p=0.007$) and Dunnet's post-hoc test confirmed that B3 was the sole breakfast to display a significant FZ decrement compared to glucose ($p<0.01$).

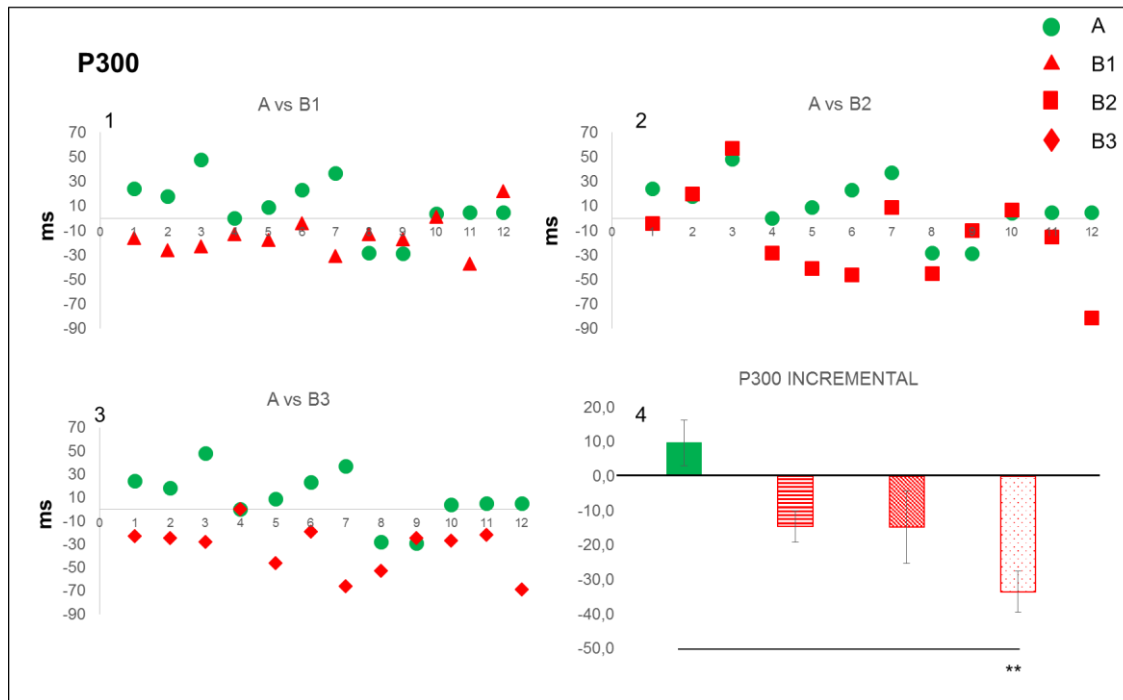


Figure 3.2a: these graphs show, for each subject, P300 incremental after consumption (P300 value post-breakfast consumption minus P300 value before breakfast consumption). A positive difference between post breakfast and fasting P300 values (ms) indicates that P300 peak is postponed after breakfast consumption. A negative value stands for a peak anticipation. Repeated Measures ANOVA with Greenhouse-Geisser correction was significant ($p=0.007$). Dunnett's post-hoc test showed that B3 was the only group to display a significant decrement of FZ with respect to Control ($p<0.01$). All data are represented as mean \pm sem. *: $p<0.05$; **: $p<0.01$.

3.3 Correlation and linear regression

Only breakfast 3 plasma ghrelin final inhibition and P300 FZ decrement (FZ latency value post breakfast consumption minus FZ latency value pre breakfast consumption) were positively correlated ($P=0.01$; $R^2=0.37$; slope coefficient=1.964, *figure 3.3a*).

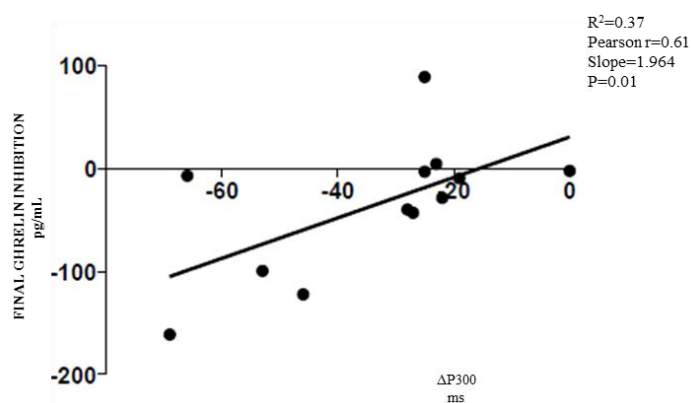


Figure 3.3a: Linear correlation between final ghrelin inhibition (Y axis) and $\Delta P300$ (X axis; $\Delta P300$ = difference between P300 FZ value post consumption and P300 FZ value pre-consumption) after B3 ingestion.

<i>Glycemic profile</i>								
	0'	15'	30'	45'	60'	75'	90'	120'
A								
Mean	77	106	124	123	109	98	94	82
SEM (±)	1	6	6	7	5	5	7	6
B1								
Mean	76	88	110	99	94	91	88	84
SEM (±)	2	4	4	4	5	3	3	1
B2								
Mean	77	95	87	77	70	75	79	81
SEM (±)	2	4	5	3	3	3	3	2
B3								
Mean	76	98	92	82	81	83	85	86
SEM (±)	1	3	5	5	5	4	4	4
<i>Insulinemic profile</i>								
	0'	15'	30'	45'	60'	75'	90'	120'
A								
Mean	5,6	25,3	37,0	43,6	37,8	29,3	27,3	12,6
SEM (±)	0,6	4,0	5,1	5,7	3,2	3,3	4,5	2,8
B1								
Mean	6,1	20,5	33,3	25,4	21,8	16,8	15,0	7,8
SEM (±)	0,7	3,5	4,7	3,0	3,6	2,7	3,0	0,6
B2								
Mean	6,4	39,5	36,3	21,9	15,6	16,8	14,6	11,8
SEM (±)	0,6	4,2	3,4	3,6	1,7	2,2	1,7	2,1
B3								
Mean	6,0	36,8	35,8	22,5	22,5	20,0	21,9	17,7
SEM (±)	0,5	2,6	4,7	3,0	2,9	3,2	3,6	3,3
<i>Ghrelin profile</i>								
	0'	15'	30'	45'	60'	75'	90'	120'
A								
Mean	415	431	402	393	404	385	393	421
SEM (±)	38	35	30	32	33	29	27	44
B1								
Mean	417	410	347	346	371	358	392	419
SEM (±)	49	43	35	32	36	40	46	46
B2								
Mean	405	378	338	328	339	354	379	382
SEM (±)	31	29	25	23	20	21	30	30
B3								
Mean	369	368	342	318	321	322	331	332
SEM (±)	28	34	28	24	26	26	27	30

Table 3.1a: table representing all data from OGTTs and breakfast test loads. All data are represented as media ± standard error of the mean.

4. DISCUSSION

Obesity rapidly became a health emergency, both in developed and in developing countries. Urbanisation, augmented or imbalanced food intake, sedentary life style, genetics and diseases affecting endocrinology system are the main culprits of this emergency. Amongst the range of different causes and therapies related to this disease, the aspects related to food properties are studied with greater and greater attention since they may hide positive or negative properties linked to obesity onset, speed-up or delay.

Regular breakfast consumption is a helpful habit to control BMI, satiety and to obtain and maintain the correct level of attention during morning, especially for children and adolescents. While metabolic and hormonal profiles have been widely studied, breakfast consumption effects on attention and cognitive performance are reported indirectly by analysing school achievements and behavioural aspects. Compared to this, studies evaluating cognitive processing of sensory information of different breakfasts are still few. Furthermore, studies tend to show chronic effects of breakfast consumption, but little is reported on acute effects. To our knowledge, this is the first time in which a study evaluated metabolic, hormonal and neural aspects all together.

Given the importance of breakfast in the daily food intake, in this study, we analysed how three commercial breakfasts when compared to glucose, differently affected metabolic response and cognitive performance via two well-known and characterised protocols: OGTT and evoked-related brain potentials monitoring [51]. The data we collected showed that those breakfasts are able to stimulate a lower glycemic response even if the tested breakfast had a greater carbohydrates content (as shown in **table 2.1d**). Notably, breakfast labelled as B3 showed the same glycemic pattern as B1 and B2, even if its carbohydrates content was greater.

In accordance with glycemic profiles, insulinemia showed a similar trend. As depicted in **figure 3.1.2a** all tested meals were able to minimize insulin excursion. Keeping an eye on food intake and weight control, this is an aid in controlling hunger, since the reported effects of high insulin peaks on appetite [52].

Furthermore, ghrelin excursions data showed how these breakfasts had the tendency to inhibit total ghrelin secretion, thus maintaining satiety regulation. What is particularly relevant in support of the latter sentence is that ghrelin inhibition was stable during the whole OGTT. In addition, while control and B1 ghrelinemia plasma level after

120minutes was the same as the basal data (A: 415 ± 38 vs 421 ± 44 B1: 417 ± 49 vs 419 ± 46), meals B2 and B3 showed a tendency in lowering ghrelin plasma levels (B2: 405 ± 31 vs 382 ± 30 B3: 369 ± 28 vs 332 ± 30) thus not in a significant fashion. These data suggest the importance of consuming breakfast with a blend in macronutrients in controlling satiety.

If suggested in a controlled dietary life style, this kind of breakfast may help reduce the food and consequent caloric intake during lunch, helping the weight loss. A model of breakfast as the one proposed in meals B2 and B3, could be an ally in fighting scarce compliance of patients undergoing a restricted dietary life style or simply hale and hearty individuals willing to follow an healthier diet. In fact, the consumption of high-palatable foods could enhance satisfaction and adherence to dietary life styles.

The control on insulin and the tendency to inhibit ghrelin exerted by tested breakfasts can certainly help and maximise this aspect. Furthermore, ghrelin is responsible not only for the quantity of food ingested, but also for its quality. High ghrelin plasma levels can lead to prefer aliments with high content in fat and energy intake [26]. As reported before, ghrelin functions go beyond appetite triggering and food carving. Since this hormone promotes adipogenesis and lipogenesis [27], a better inhibition can be helpful in preventing gain weight and the related consequences.

The electrophysiological results found in this work on acute effects, demonstrated that B3 breakfast was able to anticipate significantly P300 peak latency. Since P300 latency reflects the amount of time an individual needs to compare the deviant stimulus received with the standard ones and to shift its attention, the peak anticipation occurred after 2 hours from B3 ingestions (-33.9 ± 5.9 ms) reflects an improved cognitive performance when compared to glucose. The presence of hazelnut and chocolate in B3 meal has to be taken in account due to the presence of macronutrients of which both aliments are rich [53-54]. These macronutrients are methylxanthines and flavonoids. Xanthines, which also are a product of from caffeine metabolism, have effects on cardiovascular system, improving vascular function and lowering blood pressure [55]. Furthermore, they are able to cross the hematoencephalic barrier and binds to adenosine receptors, influencing arousal, mood and concentration [56]. Flavonoids, on the other hand, are thought to ameliorate neural activity by acting on blood flow and/or oxygen consumption [57].

As previously reported in literature, regular breakfast consumption is associated with better outcomes in school achievements and children behaviour [11]. A wide range of studies reported that poor nutritional status disturbs brain functionality [58-59] but even

if it is known that breakfast skipping is an unhealthy habit, more and more people keep skipping it. In this scenario tendency to inhibit ghrelin and breakfast composition are again advocated: since ghrelin may mediate reward responses to food, its inhibition the consequent satiety regulation and the related reward feeling may contribute in fighting scarce compliance in following a healthy diet style. The reward effect from eating something palatable and the awareness of “being more attentive” that individuals may experience after chronically consuming a particularly designed breakfast, may also potentiate this aspect.

Alongside these considerations on the interplay of ghrelin inhibition and P300 evoked related brain potential, only in B3 a significant association between final ghrelin inhibition and P300 peak anticipation was found. Even if the association cannot make us suggest that ghrelin inhibition and P300 peak anticipation are in a causal relationship, it is true that meal B3 elicited more desirable responses, from metabolic and neurofunctional profile.

All tested breakfasts (B1, B2 and B3) induced lower glycemic responses and AUCs when compared with glucose. Meal B1, composed by corn flakes and milk, elicited the highest one thus not in a significant way. This feature could suggest that the higher amount of carbohydrates present in B2 and B3 was not deleterious in respect to glycemic responses.

Another desirable facet showed by tested breakfasts is that insulinemic AUCs were contained with respect to control (see). Thus not significant, this tendency is desired due to the above-mentioned effects of high insulinemic levels, which may possibly trigger hunger and promote adiposity.

In conclusion our findings, new both in their integrative approach and the analysis of acute effects, strengthen the message of the importance of the first meal of the day. Consuming a well-balanced breakfast is a helpful tool to prevent gain weight and the subsequent dramatic consequences and to go through morning day-life challenges that require active attention and focus.

5. EU-China Partnership on Novel Strategies to Treat Diabetes from Plant-derived proteins (ECRIP)

5.1 Introduction

During the last months of my doctorate fellowship, I had the opportunity to study at the Sixth's People Hospital affiliated with Jiao Tong School of Medicine of Shanghai.

The purpose of this project, founded by European Union Delegation of China and Mongolia, is to develop novel approaches to diabetes management via the usage of nutraceuticals normally found in ingested food and to study the differences between Han population, the biggest among 56 Chinese ethnic groups, and Caucasians [60].

Literature regarding *nutraceutical* aspects of particular macronutrients found in edible vegetables or fruit is growing so much that lectures on pathological and physiological effects of some molecules are now scheduled in national and international congresses regarding treatment of obesity and even diabetes. The team I had the chance to work with during these years, as a notable example of the importance of this subject and its concern, detains a patent of nutraceutical effects of conglutin- γ , a protein found and extract from lupin, on insulin resistance. This is an example showing that researchers, alongside the effects shown in clinical field, want to know what happens in a deeper, molecular level.

These substances are important for a particular set of reasons: the law and the long, complex process regarding the commercial emission of foods and nutraceutical substances are not as strict as the ones regarding a new treatment or drug. For the above-mentioned reason, they are readily and quickly available for market and more accessible to people. Additionally, the fact that these molecules are found in common foods known and maybe appreciated more by patients or healthy individuals may help compliance to treatment.

5.1.1 Diabetes in Asia

Diabetes prevalence rose during the past years all over the world. Recorded number of 180 million patients in 1980 reached the dramatic 420 million peak in 2014, as reported by World Health Organization (WHO, 19). Developing countries are facing rapid urbanization and modernization rates and predictions are for them to display the highest incidence and prevalence of diabetes [61]. Amongst these countries, India and China share the major number of subjects with full-blown diabetes or prediabetes [61] and estimations are that these countries will share the highest number of diabetic patients worldwide [62].

In China, on which ECRIP project focuses, during past decades rapid modernization and urbanization occurred, helping obesity spread. To give an idea of how quickly this process occurred in China, the percentage of urbanization in China rose from 27.4% in 1990 to 44.9% in twenty years span [63]. Urbanization and modernization had a strong impact on Chinese population habits since they led to migration phenomena from rural areas to cities. The migration from rural areas to metropolis and modelling their life style to a more globalized one resulted in an augment in physical inactivity and in BMI, increasing overweight, obesity and diabetes [64].

However, the actors that led to increase in obesity and diabetes are not urbanization and modernization only. In fact, Chinese behavioral habits helped type 2 diabetes mellitus upraise. Eating has several meanings for Chinese culture, Han population views food consumption and sharing in context of social bonding and interaction, often involving close friends and family members. [65] Chinese population are known for eating in groups and foods are designed and cooked to be eaten communally, thus leading to the spread of rich banquets as a way to consume food. These banquets may help an unhealthy and imbalanced nutrient and caloric intake.

Alongside socio economic ones, some other features exist: Asian populations seem to be more prone in developing features related to diabetes onset. In fact, they show insulin resistance and MetS features (hypertriglyceridemia; increased abdominal or visceral fat) starting from young age, even if obesity is absent [66~69]. Strengthening these aspects, when compared to Western patients, Asian populations show the tendency to develop diabetes earlier [70].

While sharing the same dramatic rise in diabetes incidence, Asian and Caucasians diabetic patients' clinical features are not exactly the same. In literature, several studies report the differences between clinical features of type 2 diabetes mellitus in Asian populations when compared to Caucasians [70]. Asians suffering from diabetes are more prone to develop micro and macroalbuminuria when compared to Caucasians [71-72].

Furthermore, Asian patients are more prone to suffer from proteinuria, retinopathy, microalbuminuria and macroalbuminuria (45%,57%,40% and 60%, respectively) when compared to Caucasians (29%, 28%,29%, 40% and 60%) [70].

Also differences in genetic factors subsist. As an example, the protective effect on insulin resistance exerted by proliferator-activated receptor- γ (PPARG) rs1801282 SNP (Pro12Ala) seen in Western population was not reported regularly in Eastern ones [73]. Another SNP, related to T2DM development is rs7903146 on transcription-factor-7-like protein 2 (TCF7L2). However, this risk factor is not associated with T2DM in Asian populations. [74-75].

The example reported, is well explained by a monogenic form of diabetes: Maturity Onset Diabetes of the Young (MODY). Subjects affected by this particular disease are characterized by a young age at onset (usually <25 years old), are insulin independent, have first-degree relatives suffering from diabetes and lack specific Type 1 Diabetes Mellitus antibodies. Furthermore, in contrast with T2DM, BMI falls within normal range [76].

Different genes are associated with different MODY types onset. While in Caucasian populations MODY3 and MODY2, cover for the 80-85% of this particular diabetes cases [77], studies conducted in Asian countries give totally different results: as an example, a study conducted on 146 unrelated clinical MODY families in Hong Kong found out that MODY3 and MODY2 prevalence was 9 and 1% respectively [78].

These evidences highlight the differences existing between Chinese and Caucasians diabetic patients both in clinical features, complications, and in polymorphisms (SNPs), for these reasons, part of the project is to apprehend adequate and standardised protocols to characterize the population studied, in order to compare them properly and define better the outputs of the research. As part of ECRIP programme, my main aim was to understand and become confident with molecular biology techniques such as sequencing. The protocol I have learned will be discussed further in this thesis.

6 MATERIAL AND METHODS

6.1 Sanger's Reaction

The main aim of my internship in Shanghai was to learn molecular biology techniques in DNA extraction, amplification and sequencing via Sanger's reaction.

Sanger's sequencing reaction, developed in 1977, is the basis of the First-Generation Sequencing (FGS) laboratory equipment [80]. This technique is to date the gold standard for detecting unknown mutations in specific DNA Regions of Interest (ROI). The rationale of this protocol is to introduce dideoxynucleotides (ddNTPs) into a DNA synthesis reaction. These ddNTPs lack a hydroxyl –OH group in 3' position and their inclusion in the DNA strand blocks further synthesis by DNA polymerase [80].

If, for instance, a DNA template undergoes amplification with a traditional PCR mix containing primers, ions, MgCl₂, DNA polymerase and deoxynucleotides (dNTPs), the whole ROI comprised in between the primers will be synthesized. However, if a traditional PCR mix contains ddNTPS-A (dideoxynucleotides Adenine), every amplicon synthesis will be randomly blocked at -A positions during strand elongation, generating a mixture of DNA fragments, with different lengths.

It is possible to read and analyse the sequences of ddNTPS-A bases on these fragments using denaturing acrylamide gel. This is due to the different length of the amplicons and to the high separating power of the acrylamide net. If every base follow the same protocol, it is possible to read the whole ROI.

During the following years, and after the introduction of Polymerase Chain Reaction (PCR) by Kelis Mullis, companies and researchers developed new techniques and technologies to obtain better and faster sequencing results. Accompanied by this vision, market saw the introduction of automated sequencers and new chemistries. Between the several different protocols now available to sequence a particular ROI, the one I used relays on *dye terminators chemistry*.

This protocol differs from the original one since the reaction mix used contains every ddNTPs, avoiding the usage of four different mixes for one sample. Four different fluorochromes label the four different ddNTPs, so that every base is associated with a specific fluorescence. Thanks to this, it is possible to generate, in one single reaction tube, differently labelled amplicons with different lengths, according to the random inclusion

of marked ddNTPs during the synthesis process lead by DNA polymerase. **Figure 6.1a** gives a schematic representation of the protocol rationale.

The sample is then analysed via dedicated equipment to generate an *electropherogram* as shown in **figure 6.1b**.

Samples, running on a polymer gel, generate the electropherogram. As during a standard electrophoresis, PCR products run through the gel according to their length (short products run quicker than the longer ones, since they easily cross the net created by the gel polymer) and specific detector records their fluorescence and translates it into peaks, writing ROI sequence.

DNA sequencing is a multistage technique and to get meaningful and trustful results, several steps must precede sequence analysis. The steps that will be discussed in detail later are DNA extraction, Region of Interest amplification, purification, proper Sanger's reaction, purification and reading.

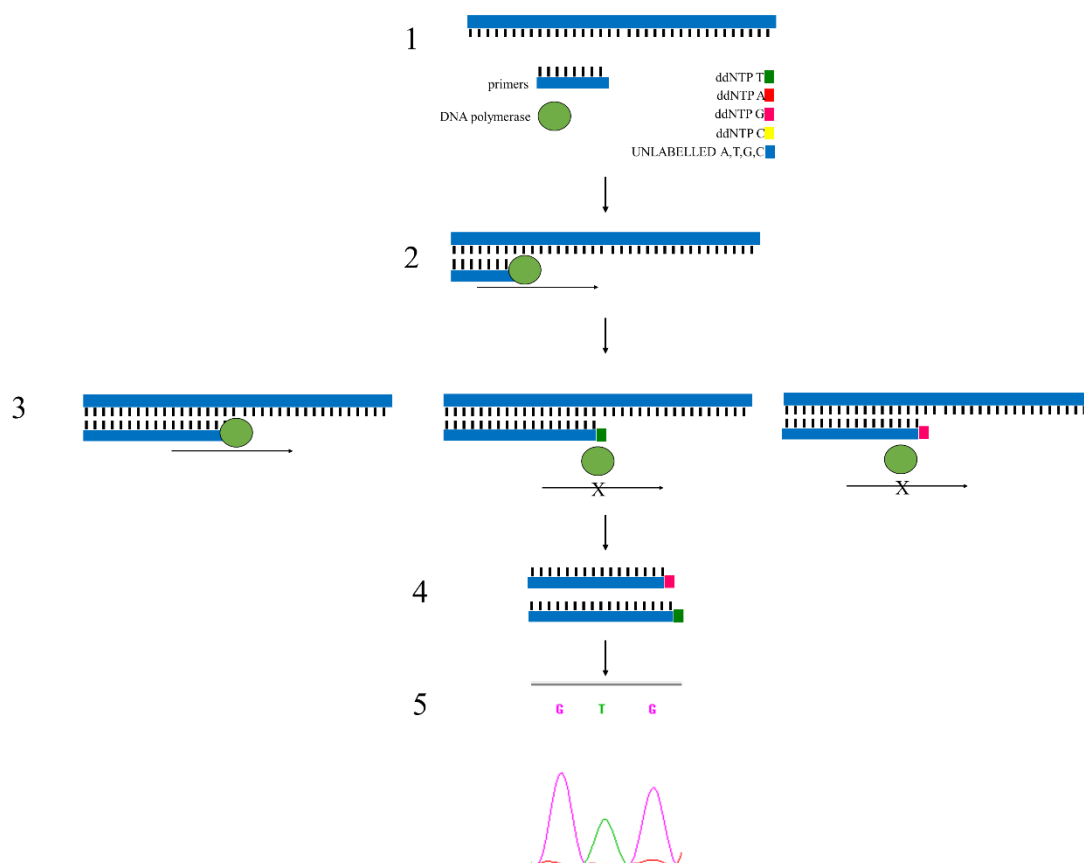


Figure 6.1a: schematic representation of Sanger's Reaction with terminal dye chemistry. DNA template (long blue line), previously amplified via classical PCR, undergoes another PCR cycle with forward or reverse primer (short blue line), DNA polymerase, dNTPs and fluorochrome-labelled ddNTPs (1). During this step in which DNA polymerase binds to hybrid template and primer, starts elongation (2). While synthesizing the new DNA strand, the enzyme randomly picks up dNTPs or ddNTPs (3). If a dNTP is added to the sequence (3-left), the enzyme continues elongation. Inversely, if a ddNTP is added the synthesis reaction stops (3-center, right).

This generates a series of amplicons different in length and labelled according to the last base added (4) After Sanger's reaction, samples undergo a gel electrophoresis in dedicated equipments, which read the length and fluorescence of each amplicon fragment and give as a result an electropherogram (5).

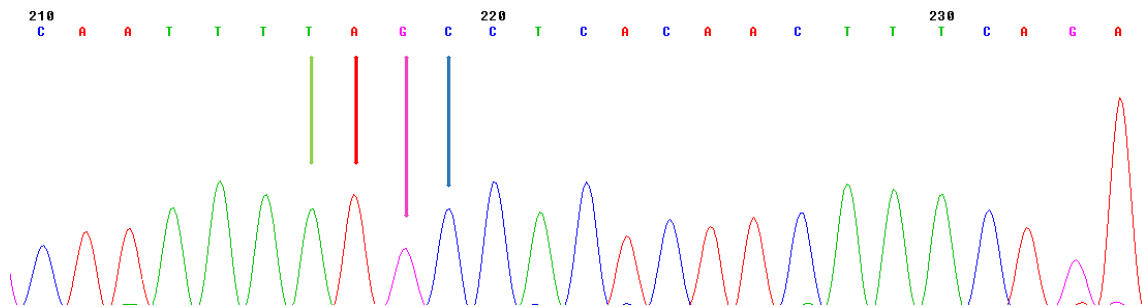


Figure 6.1b: an electropherogram. The software reads amplicon fragments as they pass by during electrophoresis and renders their fluorescence as peaks. The result is the sequence of the region of interest. Each colour is matched with a different nucleotide (arrows).

6.1.1 DNA Extraction

DNA extraction is a crucial step in sequencing reaction analysis, since maximum purity and quality are mandatory features in order to obtain a fully readable electropherogram. To extract DNA, a standard commercial kit is used: QIAGEN DNeasy kit code 69054.

The above-mentioned kit spoils a patented column method to extract DNA from cells and elute it. The sample used is the buffy coat generated after centrifugation of peripheral blood collected in vacutainer tubes containing EDTA. The presence of EDTA as anticoagulant prevents subsequent amplification reactions inhibition.

This extraction method is quick, allows the manual processing of several samples at the same time, and is summarised in *figure 6.1.1a*. The cellular components of buffy coat are firstly lysed thanks a brief vortexing and incubation with proteinase K. The lysate is then mixed with ethanol and transferred to the spin columns.

The column then undergoes three centrifugations steps with different buffers in order to wash out impurities and proteins. The final step is an elution in a small amount of Nuclease-free water (from 20 to 50 μ L).

After this extraction, samples' quantity and purity is observed trough spectrophotometer analysis. In fact, if the sample is old, stored in bad conditions or poorly extracted, the quantity may be affected. Furthermore, during the extraction process, extraction reagents (especially ethanol and isocyanil guanine) may pollute DNA and interfere with later protocols.

The spectrophotometer generates a light beam at different wavelengths (280nm, 260nm, 230nm) that pass through the sample, reads the different absorbance levels, expressed in optical densities (OD) and uses them to calculate DNA quantity and pollution:

- A280nm: the software utilises the general rule that 1 OD corresponds to 40ng/ μ L of DNA and adjusts it by a specific distance correction factor to obtain DNA concentration.

- A260nm: this value is used to verify the presence of proteins in the eluted samples. The ratio between A280 and A260 gives us a value that must fall within 2-2.10, thus indicating a sample containing DNA only

-A230nm: thanks to this, it is possible to state whether the sample pollution by ethanol, isocyanil guanine or other agents occurred. The ratio between A280 and A230 must fall, again, within a 2-2.10 range. The lower the value obtained, the more polluted is the sample.

Samples failing to meet these conditions need to be extracted again, since further reactions may be influenced.

- Add buffy coat (50-100 μ L)
or Whole Blood (5-10 μ L)
- Add proteinase K 20 μ L
- Add PBS (adjust volume to 220 μ L)



- Add 200 μ L Buffer AL
- Incubate at 56° for 10min



- Add 200 μ L of ethanol (96-100%)
- Vortex
- Transfer to a spin column placed in a collection tube



- Centrifuge $\geq 6000 \times g$ 1minute
- Place the spin column in a new collection tube
- Add 500 μ L of Buffer AW1 into the column
- Centrifuge $\geq 6000 \times g$ 1minute
- Discard the flow through
- Add 500 μ L of Buffer AW2 into the column
- Centrifuge $\geq 8000 \times g$ 3minutes
- Put the column in a new 1.5 collection tube
- Add 200 μ L of Buffer AE
- Incubate for 1 minute at room temperature
- Centrifuge $\geq 6000 \times g$ 1minute

Figure 6.1.1a: representation of experimental protocol used to extract DNA from blood samples.

6.1.2 Region Of Interest Amplification

To sequence correctly the region of interest, it is important to fragment it virtually. The sequencing reaction must have an amplicon which is no longer than 500bp, so it is necessary to design different set of primers able to cover all the sequence of interest.

Another important aspect of primer design is that after sequencing reaction has occurred, due to interferences, roughly from 50 to 100bp at the beginning of the amplified region are usually lost. To avoid loss of information, primers should hybrid to genome 50 to 100bp before actual ROI. A sample of a single exon gene is reported in *figure 6.1.2a*.



Figure 6.1.2a: A hypothetical gene composed by one exon (dark green rectangle) is virtually fragmented by three set of primers: red) set one; yellow) set two; orange) set three. Primers have to be placed in order to cover all the region of interest. Note how the three sets overlaps and that set one and set three fall outside the region of interest (small light green rectangles).

Samples then undergo a standard PCR cycle, according to the polymerase enzyme used and the melting temperature of each primer set (*figure 6.3b*).

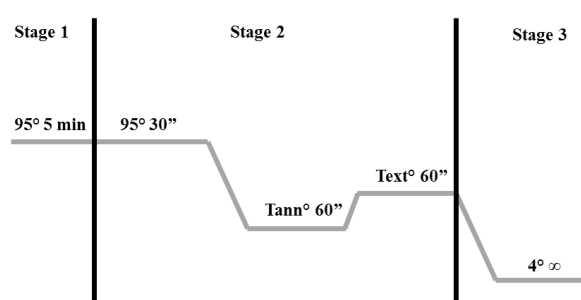


Figure 6.3b: PCR protocol used. Tann: Annealing temperature; Text: extension temperature. Tann has to meet the requirement of the specific primers set used, while Text matches the needs of the DNA polymerase used.

As DNA sequencing requires high starting material quality and purity, it is binding to check the amplification via electrophoresis on acrylamide gels. Frederik M et al describe the standard protocol used to synthesize acrylamide gels and run samples in Current Protocols in Molecular Biology [81]. This process allows to visually identify the

amplicons produced during PCR reaction comparing the bands with the ones obtained by a simultaneously ran molecular weight. This sample generates a “ladder” of bands of known length. The criteria used to validate ROI amplification were:

- amplified length in accordance with expected length;
- presence of only one band

Fulfilling the above-mentioned conditions allow certifying the accuracy of the amplification and its purity. Furthermore, it is possible to check the presence/absence of unwanted amplified regions and adjust PCR conditions accordingly.

As for DNA extraction, only samples that go on to the next stage: purification.

6.1.3 Amplicons Purification

After PCR amplification products validation via gel electrophoresis, the successive step is purification. In fact, after the first PCR, amplicons are diluted in the reaction mix used for the first PCR. This mix contains unused dNTPs, magnesium, primers, other buffer ingredients and ions. Since the presence of these factors can nullify further sequencing reaction, purification is needed.

To do this, several commercial kits are available. The protocol does not differ from DNA extraction and purification one above-described since the purpose is to hold DNA in a membrane while the unwanted buffers, salts or molecules are washed away by centrifugation steps.

The protocol used is from the commercially available kit from LIFEFENG code DK404-02. Following the protocol provided with the kit, appropriate wash buffers are added all together to PCR amplification product to a spin-column membrane, which undergoes subsequent centrifugation steps.

Finally, Nuclease-Free water is added to the column which is centrifuged, in order to release the copies of amplified DNA.

At this stage, amplified samples are suitable for the next step, which is the actual Sanger's sequencing reaction.

6.1.4 Sanger's Sequencing Reaction

During this modified PCR protocol, each sample is assigned to two reaction tubes, for forward primer and reverse one. Forward and reverse primers separate amplification has some advantages.

Firstly, each fragment amplified on one primer can be read on the other one thus allowing confirmation of variations in genome. Secondly, the amplification of both forward and reverse primer in the same tube leads to the presence of amplicon fragments of the same length but belonging to different regions of the tested sequence. Co-presence of these fragments in the tube generates inconsistent and mixed data, thus the need to separate forward and reverse reaction.

An appropriate reaction mix is prepared to the purpose. The mix contains the typical dNTPs, enzyme, ions and buffers of PCR reaction but with two main differences: the presence of ddNTPs labelled with fluorochromes and the usage of a single primer (forward or reverse) instead of the usually combined set.

The samples are added to the mixes and undergo PCR cycles to synthesize amplicon fragments, according to the specific features of DNA polymerase enzyme used.

6.1.5 Second Amplicons Purification

The final step before fragments reading is another purification. Even if samples end their amplification steps after Sanger's reaction, the presence of enzymes and unused labelled ddNTPs interferes with sequence reading.

This step differs from the first purification and relies on BigDye XTerminator® Purification kit (ThermoFisher Scientific, code 4376486) technology. The purifying solution is a mixture that allows the isolation of the above-mentioned unwanted mix remaining and fragments stabilization. The mixture, composed by two reagents (XTerminator™ Solution and SAM™ Solution) captures unused ddNTPs and buffer from the post-sequencing reaction and stabilizes the sample for subsequent reading.

Samples are added at the bottom of a 96 multiwell plate, and according to kit instructions, in each well are added 45µL of SAM™ Solution and 10µL of XTerminator™ Solution. The plate is then covered with an adhesive film and undergoes vortexing for 30 minutes, in dark environment. This allows the reagents to free the solution from unused ddNTPs and buffers. After that, the plate is centrifuged briefly and analysed on dedicated instruments for electrophoresis and reading. In this case, Applied Biosystems 3500xL Genetic Analyser was used (ThermoFisher Scientific).

6.1.6 Sequence Reading and Analysis

After the sample run into the polymer amplicons lengths and fluorescence, which are read through detectors, are translated into peaks (*figure 6.1b*) in an electropherogram.

Before reading, the electropherograms produced undergo a quick screening to assess their quality. *Figures 6.1.6a* provides an example of electropherograms with different quality levels. Only results allowing unambiguous reading have to be analysed. In fact, the presence of mixed peaks invalidates proper analysis (*figure 6.1.6a, panels B-C*). Samples failing quality control needs to be repeated.

Good quality samples are read by scrolling the sequence, from left to right and looking for double peaks *figure 6.1.6b* gives an example of how a heterozygous SNP/mutation presence emerge. In this way, it is possible to screen the sequence for heterozygous alterations only.

To obtain data on homozygous alterations, insertions or deletions we use dedicate software. They allow the comparison between the sequence to analyse and a reference one by aligning them and reporting lack of bases, insertions, deletions or homozygous SNPs/mutations. The reassuring presence of software able to read and compare sequences must not be overrated, in fact, only a trained eye can assess electropherogram quality and distinguish between real peaks and disturbances in a proper manner.

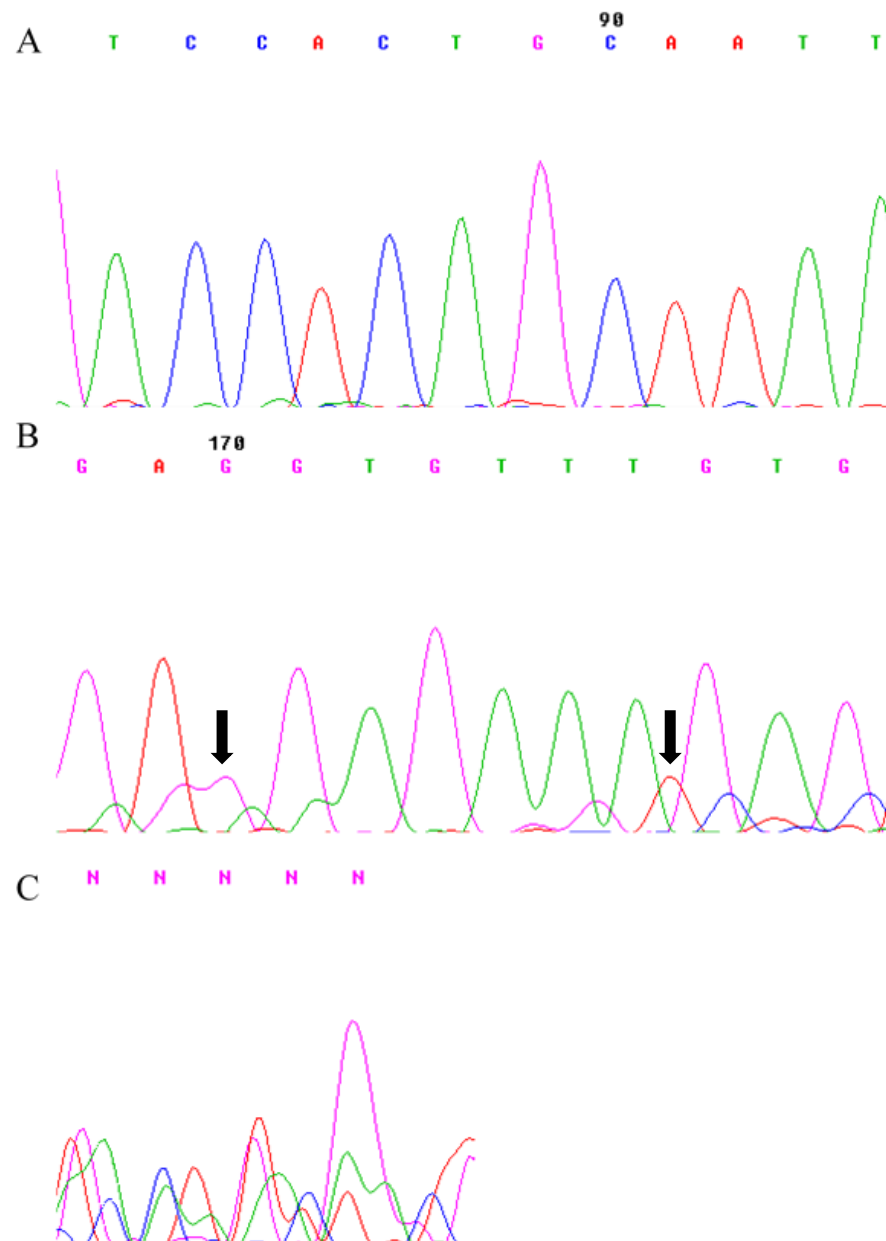


Figure 6.1.6a: Different reading analysis outcomes. **Panel A** shows a good quality electropherogram, since peaks are divided and the sequence can be read clearly. Furthermore, the absence of background disturbances allows determining the presence/absence of heterozygous peaks. **Panel B** shows this characteristic: the presence of disturbance peaks (black arrows) make it impossible to determine the presence/absence of heterozygous peaks. Disturbances may be so severe that the whole sequence is impossible to read (**panel C**).

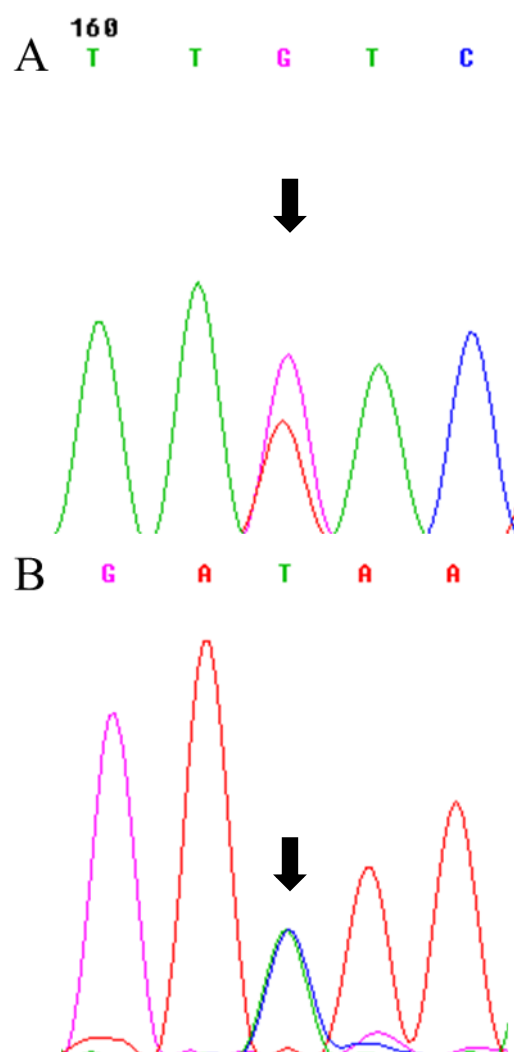


Figure 6.1.6b: SNP variation read on an electropherogram. **Panel A** shows a 5'-3' strand carrying a heterozygous SNP on position G. Differently from the previous well defined peaks, the third one is mixed with another one, proving the presence of two different bases on that position. **Panel B** shows the same SNP, of the same proband, on 3'-5' strand. The simultaneous presence of the same SNP on both strands validates it.

7.1 DISCUSSION

Sequencing become during past years a toll to diagnose and discover diseases, to study populations genetic and to characterize specific aspects of patients and healthy individuals.

During the internship in Shanghai, I had the chance to become confident with First Generation Sequencing and to gather the required skills to do and validate such experiments.

While powerful and adaptable, this molecular biology protocol needs high standardization in every step, since the output of the final reading and analysis relays on the quality of previous stages. A poor DNA extraction, for example, can lead to failed PCR amplification and imprecise primer design is mandatory to reach reliable results. Furthermore, skipping amplicons purification between stages can affect subsequent reactions and reading.

For these reasons, it is important to recognize what could have gone wrong while looking at a poor or failed electropherogram. An accurate analysis of the latter can tell a lot about the quality of the stages and may help troubleshooting: in fact, different systematic or random errors lead to different electropherograms.

If the electropherogram is composed by several, high peaks, overlapping one with another, chances are that the mix contained both forward and reverse primer and not just only one. If peaks are extremely high, out of the detection range of the instrument, the amount of initial DNA was probably too high, so one should check extracted DNA concentration and dilution.

Repeated, medium-high peaks could be interferences due to poor purification system. These peaks are particularly tricky because their fluorescence value can be as high as normal peaks and the software may mistake them for actual peaks. In this situation, further analysis to screen for insertion or deletion, will lead to dramatic false positive results.

Even the position of failed samples needs attention; when different samples, performed on different runs but in the same well give negative results, the capillary system can be damaged, expired or poorly placed on the instrument. Case in point of the amount of elements that can generate a poor quality electropherogram.

Though leading to further costs due to the repetition of the analysis, and that a good result is the main aim of sequencing reaction, the information given by an unsatisfactory electropherogram may be at least as useful as the one given by a good one. Thorough accurate troubleshooting analysis it is possible to act on the aspect that led to failed result maybe revealing a step in the protocol that should be reviewed. This allows to keep going towards cleaner and clearer results.

Even if softwares are necessarily in sequencing analysis, they still lack the artificial intelligence needed to discern between peaks and disturbances, for example. The only

means to assess the quality of a good run and to evaluate critical points is the trained human eye.

REFERENCES

1. Global report on diabetes. World Health Organization. Geneva, 2016
2. WHO Expert consultation. Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. *Lancet*. 2004 Jan 10; 363(9493):157-63
3. Verma S. et al. Obesity and diabetes: An update. *Diabetes Metab Syndr*. 2016 Jun 17. pii: S1871- 021(16)
4. Solange S. Pereira, Jacqueline I. Alvarez-Leite, Low-Grade Inflammation, Obesity, and Diabetes. *Metabolism* (R Pasquali, Section Editor) *Current Obesity Reports* (2014); 3(4): 422–431.
5. European Commission. Eurostat - Statistics Explained. Overweight and obesity – BMI statistics 2008. http://ec.europa.eu/eurostat/statisticsexplained/index.php/Overweight_and_obesity_-_BMI_statistics
6. Valsecchi et al. Gut Microbiota and Obesity. *J Clin Gastroenterol* _ Volume 50, Supp. 2, November/December 2016
7. Greenwood, J.L., Stanford, J.B., 2008. Preventing or improving obesity by addressing specific eating patterns. *J. Am. Board Fam. Med.* 21, 135–140.
8. Horikawa C. et al. Skipping breakfast and prevalence of overweight and obesity in Asian and Pacific regions: A meta-analysis. *Preventive Medicine* 53 (2011) 260–267
9. Farshchi, H.R., et al., 2005. Deleterious effects of omitting breakfast on insulin sensitivity and fasting lipid profiles in healthy lean women. *Am. J. Clin. Nutr.* 81, 388–396.
10. Adolphus et al. The effects of breakfast on behavior and academic performance in children and adolescents. *Front Human Neurosci*. August 2013 | Volume 7 | Article 425 |
11. Ni Mhurchu et al. Effects of a free school breakfast programme on school attendance, achievement, psychosocial function, and nutrition: a stepped wedge cluster randomised trial *BMC Public Health* 2010, 10:738
12. D. Benton et al. The influence of the glycaemic load of breakfast on the behaviour of children in school *Physiology & Behavior* 92 (2007) 717–724
13. Benton D, Ruffin M-P, Lassel T, Nabb S, Messaoud N, Vinoy S, et al. The delivery rate of dietary carbohydrates affects cognitive performances in both rats and humans. *Psychopharmacology* 2003;166:86–90.
14. Nabb S, Benton D. The influence on cognition and mood of the interaction between the macro-nutrient content of breakfast and glucose tolerance. *Physiol Behav* 2006;87:16–23.
15. Chugani HT. A critical period of brain development: studies of cerebral glucose utilization with PET. *Prev Med* 1998;27:184–8.
16. Kalhan SC, Kilic I. Carbohydrate as nutrient in the infant and child: range of acceptable intake. *Eur J Clin Nutr* 1999;53(Suppl 1):S94–S100.
17. Hayley Y et al. The glycemic load of meals, cognition and mood in middle and older aged adults with differences in glucose tolerance: A randomized trial. *e-SPEN Journal* 9 (2014) e147ee154
18. Sandercock GRH, Voss C, Dye L: Associations between habitual school-day breakfast consumption, body mass index, physical activity and cardiorespiratory fitness in English schoolchildren. *European Journal of Clinical Nutrition* 2010, 64:1086-1092.
19. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone–releasing acylated peptide from stomach. *Nature* 1999;402:656–60.
20. De Vriese C. et al. Focus on the short- and long-term effects of ghrelin on energy homeostasis. *Nutrition* 26 (2010) 579–584
21. Laviano A, Mari A 2014 Homodimerization and heterodimerization of the ghrelin receptor. In: Portelli J, Smolders I (eds), *Central functions of the ghrelin receptor*. Humana Press, Springer Science New York; pp, 21-34.
22. Mihalache L et al. Effects of ghrelin in energy balance and body weight homeostasis. *HORMONES* 2016, 15(2):186-196
23. Solomou S, Korbonits M, 2014 The role of ghrelin in weight-regulation disorders: Implications in clinical practice. *Hormones (Athens)* 13: 458-475.
24. Blom WA, de Graaf C, Lluich A, Stafleu A, Schaafsma G, Hendriks HF. Postprandial ghrelin responses are associated with the intermeal interval in time-blinded normal weight men, but not in obese men. *Physiol Behav* 2009;96:742–8.

25. M. Tschöp et al. Post-prandial decrease of circulating human ghrelin levels. *J. Endocrinol. Invest.* 24:RC19-RC21, 2001
26. Shimbara T, Mondal MS, Kawagoe T, et al, 2004 Central administration of ghrelin preferentially enhances fat ingestion. *Neurosci Lett* 369: 75-79.
27. Delporte C, 2013 Structure and physiological actions of ghrelin. *Scientifica (Cairo)* 2013: 518909.
28. Chen CY, Asakawa A, Fujimiya M, Lee SD, Inui A, 2009 Ghrelin gene products and the regulation of food intake and gut motility. *Pharmacol Rev* 61: 430-481.
29. Lim CT, Kola B, Korbonsits M, 2011 The ghrelin/GOAT/ GHS-R system and energy metabolism. *Rev Endocr Metab Disord* 12: 173-186.
30. Anderson KA, Ribar TJ, Lin F, et al, 2008 Hypothalamic CaMKK2 contributes to the regulation of energy balance. *Cell Metab* 7: 377-388.
31. Dien J et al. Parsing the late positive complex: Mental chronometry and the ERP components that inhabit the neighborhood of the P300. *Psychophysiology*, 41 (2004), 665–678.
32. Polich J. Updating P300: an Integrative theory of P3a and P3b. *Clin Neurophysiol.* 2007 October ; 118(10): 2128–2148
33. 2a Cywicz YM, Friedman D, Rothstein M, An ERP. Developmental study of repetition priming by auditory novel stimuli. *Psychophysiology* 1996;33:680±90.
34. 2b Knight RT. Decreased response to novel stimuli after prefrontal lesions in man. *Electroencephalogr Clin Neurophysiol* 1984;59:9±20.
35. C Luria AR. The working brain. New York: Basic Books, 1973. D Sokolov EN. Perception and the conditioned re⁻ex. Oxford, UK: Pergamon Press, 1963.
36. E Kagan J. Galen's prophecy. New York: Basic Books, 1994.
37. F Polich, J., Ladish, C., & Bloom, F. E. (1990). P300 assessment of early Alzheimer's disease. *Electroencephalography and Clinical Neurophysiology*, 77, 179–189.
38. Friedman et al. The novelty P3: an event-related brain potential (ERP) sign of the brain's evaluation of novelty. *Neuroscience and Biobehavioral Reviews* 25 (2001) 355±373
39. Sokolov EN. Perception and the conditioned reflex. Oxford, UK:Pergamon Press, 1963
40. Kaplan RJ, Greenwood CE, Winocur G, Wolever TM. Cognitive performance is associated with glucose regulation in healthy elderly persons and can be enhanced with glucose and dietary carbohydrates. *Am J Clin Nutr* 2000;72:825–36.
41. Sutton S, Braren M, Zubin J, John ER. Evoked potential correlates of stimulus uncertainty. *Science* 1965;150:1187±8.
42. Mecklinger A, Opitz B, Friederici AD. Semantic aspects of novelty detection in humans. *Neurosci Lett* 1997;235:65±68.
43. SchroËger E. A neural mechanism for involuntary attention shifts to changes in auditory stimulation. *J Cogn Neurosci* 1996;8:527±39.
44. Gaeta H, Friedman D, Ritter W, Cheng JE. An event-related potential evaluation of involuntary attentional shifts in the young and elderly. *Psychol Aging* 2001;16:55±68.
45. G Kropotov JD, Alho K, Naatanen R, Ponomarev VA, Kropotova OV, Anichkov AD, Nechaev VB. Human auditory-cortex mechanisms of pre-attentive sound discrimination. *Neuroscience Letters* 2000;280:87±90.
46. Knight RT, Scabini D, Woods DL, Calyworth CC. Contributions of temporal-parietal junction to the human auditory P3. *Brain Res* 1989;502:109±16.
47. H NaËaËtaËnen R. The role of attention in auditory information processing as revealed by event-related potentials and other measures of cognitive function. *Behav Brain Sci* 1990;13:201±88.
48. I Polich, J., & Martin, S. (1992). P300, cognitive capability, and personality: A correlational study of university undergraduates. *Personality and Individual Differences*, 13, 533–543.
49. L Näätänen R. The role of attention in auditory information processing as revealed by event-related potentials and other measures of cognitive function. *Behav Brain Sci* 1990;13:201±88.
50. M Näätänen R. Attention and brain function. Hillsdale: Lawrence Earlbaum Associates, 1992.
51. Maurits NM, et al. P300 component identification in auditory oddball and novel paradigms using source analysis techniques: reduced latency variability in

- the elderly. *J Clin Neurophysiol* 2005;22:166–75.
52. Rodin J. Insulin levels, hunger, and food intake: an example of feedback loops in body weight regulation. *Health Psychol.* 1985;4(1):1-24
 53. Alasalvar C. Review of nut phytochemicals, fat-soluble bioactives, antioxidant components and health effects. *Br J Nutr* 2015 Apr;113 Suppl 2:S68-78.
 54. ME Alanon et al. Assessment of flavonol stereoisomers and caffeine and theobromine content in commercial chocolates. *Food Chem.* 208 (2016) 177-184
 55. D. Grassi, G. Desideri, S. Necozione, G.P. di, R. Barnabei, L. Allegaert, et al., Cocoa consumption dose-dependently improves flow-mediated dilation and arterial stiffness decreasing blood pressure in healthy individuals, *J Hypertens* 33 (2015) 294–303.
 56. Nehlig, A. Is caffeine a cognitive enhancer? *J. Alzheimer's Dis.* 2010, 2, S85–S94
 57. Franco R. Health benefits of Methylxanthines in Cacao and Chocolate. *Nutrients* 2013, 5, 4159-4173; doi:10.3390/nu5104159
 58. Scrimshaw N: Malnutrition, brain development, learning and behaviour. *Nutrition Research* 1998, 18(2):351-379.
 59. Leslie J, Jamison T: Health and nutrition considerations in education planning 1. Educational consequences of health problems among school-age children. *Food and Nutrition Bulletin* 1990, 12:191-203.
 60. Jian-wei Xu et al. Use of Bed Nets and Factors That Influence Bed Net Use among Jinuo Ethnic Minority in Southern China. *PLoS ONE* 9(7): e103780. doi:10.1371/journal.pone.0103780
 61. Ramachandran A. et al. Diabetes in Asia. *Lancet* 2010; 375: 408–18
 62. Wild S, Roglic G, Green A, Sicree R, King G. Global prevalence of diabetes. Estimates for the year 2000 and projections for 2030. *Diabetes Care* 2004; 27: 1047–52.
 63. UN. World population prospects: the 2007 revision population database. <http://esa.un.org/unup> (accessed April 10, 2009).
 64. Ramachandran A, Snehalatha C, Baskar AD, et al. Temporal changes in prevalence of diabetes and impaired glucose tolerance associated with lifestyle transition occurring in the rural population in India. *Diabetologia* 2004; 47: 860–65.
 65. Abate N, Chandalia M, Snell PG, Grundy SM. Adipose tissue metabolites and insulin resistance in nondiabetic Asian Indian men. *J Clin Endocrinol Metab* 2004; 89: 2750–55.
 66. Raji A, Seely EW, Arky RA, Simonson DC. Body fat distribution and insulin resistance in healthy Asian Indians and Caucasians. *J Clin Endocrinol Metab* 2001; 86: 5366–71.
 67. Snehalatha C, Sivasankari S, Satyavani K, Vijay V, Ramachandran A. Postprandial hypertriglyceridaemia in treated type 2 diabetic subjects—the role of dietary components. *Diabetes Res Clin Pract* 2000; 48: 57–60.
 68. Misra A, Pandey RM, Ramadevi J, Sharma R, Vikram NK, Khanna N. High prevalence of diabetes, obesity and dyslipidemia in urban slum population in northern India. *Int J Obes Relat Metab Disord* 2001; 125:1722–29.
 69. Zhang P, Zhang X, Brown J, Vistisen D, Sicree R, Shaw J, Nichols G. Global healthcare expenditure on diabetes for 2010 and 2030. *Diabetes Res Clin Pract.* 2010;87:293-301. doi:10.1016/j.diabres.2010.12.025.
 70. Kong, A. P. S. et al. Diabetes and its comorbidities—where East meets West. *Nat. Rev. Endocrinol.* 9, 537–547 (2013)
 71. Wu, A. Y. et al. An alarmingly high prevalence of diabetic nephropathy in Asian type 2 diabetic patients: the MicroAlbuminuria Prevalence (MAP) Study. *Diabetologia* 48, 17–26 (2005).
 72. Parving, H. H. et al. Prevalence and risk factors for microalbuminuria in a referred cohort of type II diabetic patients: a global perspective. *Kidney Int.* 69, 2057–2063 (2006).
 73. Altshuler D, Hirschhorn JN, Klannemark M, et al. The common PPAR gamma Pro12 Ala polymorphism is associated with decreased risk of type 2 diabetes. *Nat Genet* 2000; 26: 76–80.
 74. Chang YC, Chang TJ, Jiang YD et al. Association study of the genetic polymorphisms of the transcription factor 7-like 2 (TCF7L2) gene and type 2 diabetes in the Chinese population. *Diabetes* 2007; 56: 2631–37.
 75. Ng MC, Park KS, Oh B, et al. Implication of genetic variants near TCF7L2, SLC30A8, HHEX, CDKAL1, CDKN2A/B, IGF2BP2, and FTO in type 2 diabetes and obesity in 6,719 Asians. *Diabetes* 2008; 57:2226–33.
 76. Amed S et al. Maturity-Onset Diabetes of the Young (MODY): Making the Right

- Diagnosis to Optimize Treatment. Can J Diabetes. 2016 Oct;40(5):449-454.
77. Shields B, Hicks S, Shepherd M, Colclough K, Hattersley A, Ellard S. Maturity-onset diabetes of the young (MODY): how many cases are we missing? *Diabetologia*. 2010;53:2504–2508
 78. [Xu JY](#) et al. Genetic and clinical characteristics of maturity-onset diabetes of the young in Chinese patients. 1. *Eur J Hum Genet*. 2005 Apr;13(4):422-7.
 79. Shereen Giap Kah Goh et al. Evolution of diabetes management in the 21st century: the contribution of quality of life measurement in Asians. *Asia Pac J Clin Nutr* 2015;24(2):190-198
 80. Sanger F et al. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* Vol. 74, No. 12, pp. 5463-5467, December 1977
 81. Frederik M. et al. *Current Protocols in Molecular Biology*. Copyright © 2003 John Wiley & Sons, Inc.